SUPPLEMENTAL MATERIAL

CD84 links T cell and platelet activity in cerebral thrombo-inflammation in acute stroke

Short title: CD84 in ischemic stroke

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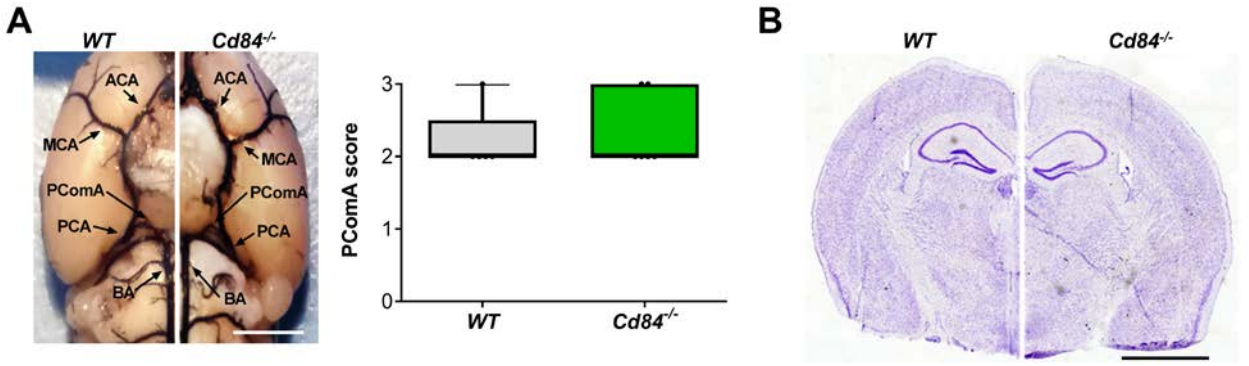
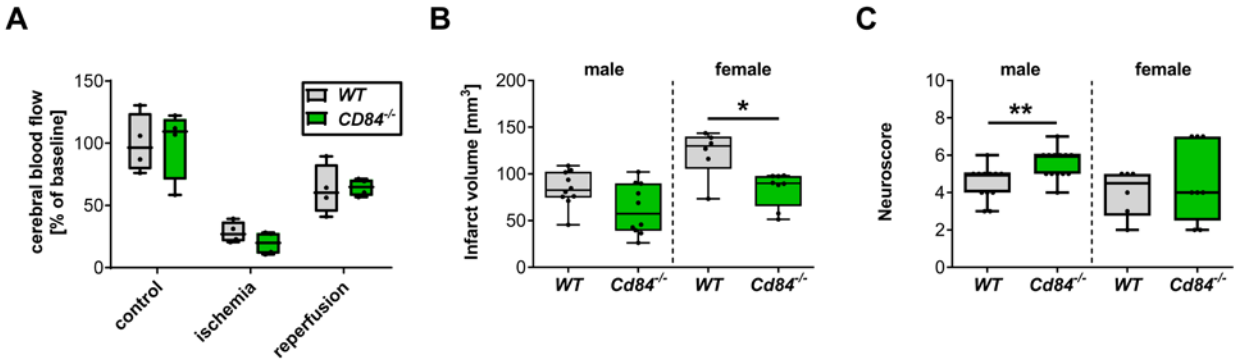
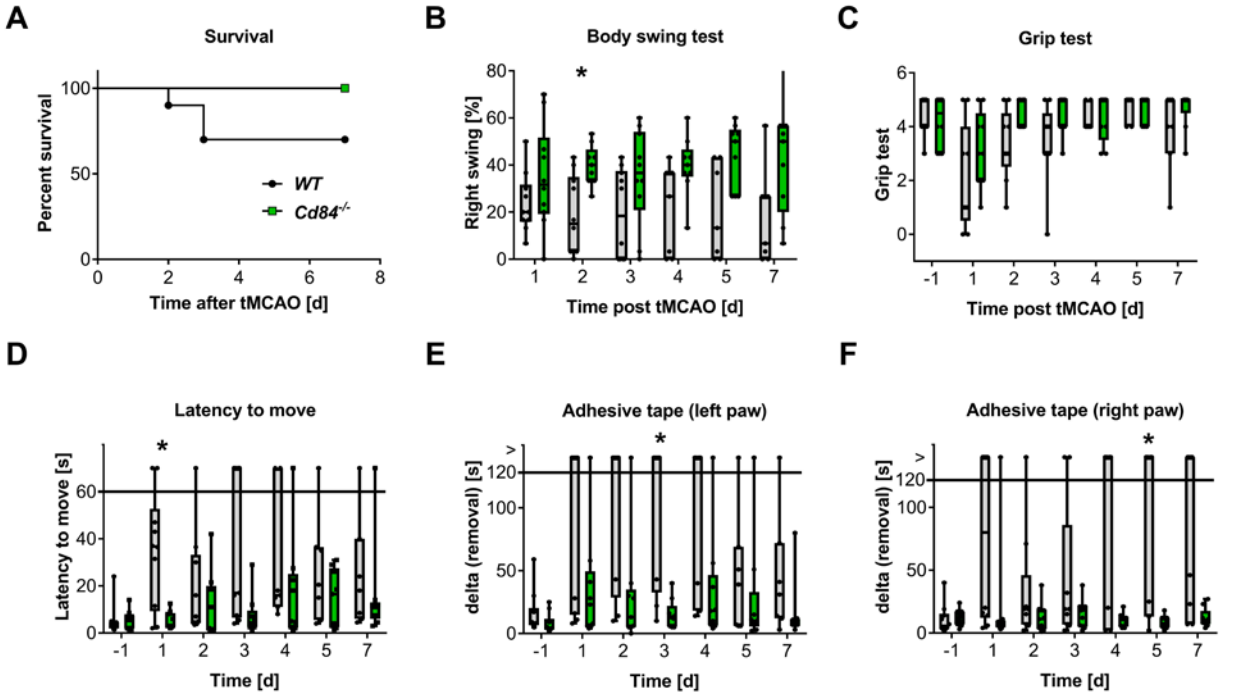


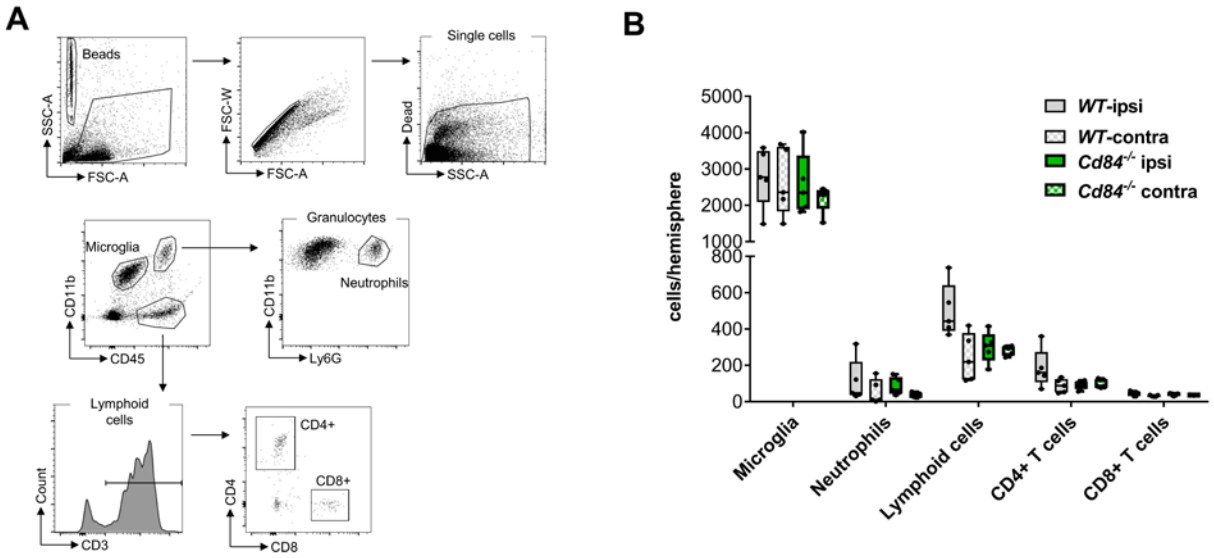
Online Figure I. Unaltered brain morphology in CD84-deficient mice. (**A**) Representative ink-stained brains of *WT* (n = 5) and *Cd84*$^{-/-}$ mice (n = 6) and corresponding PComA (posterior communicating artery) scores are presented as median (p > 0.99 using Mann Whitney test). (**B**) Nissl staining of brain slices from *WT* and *Cd84*$^{-/-}$ mice. Scale bar 0.25 cm.



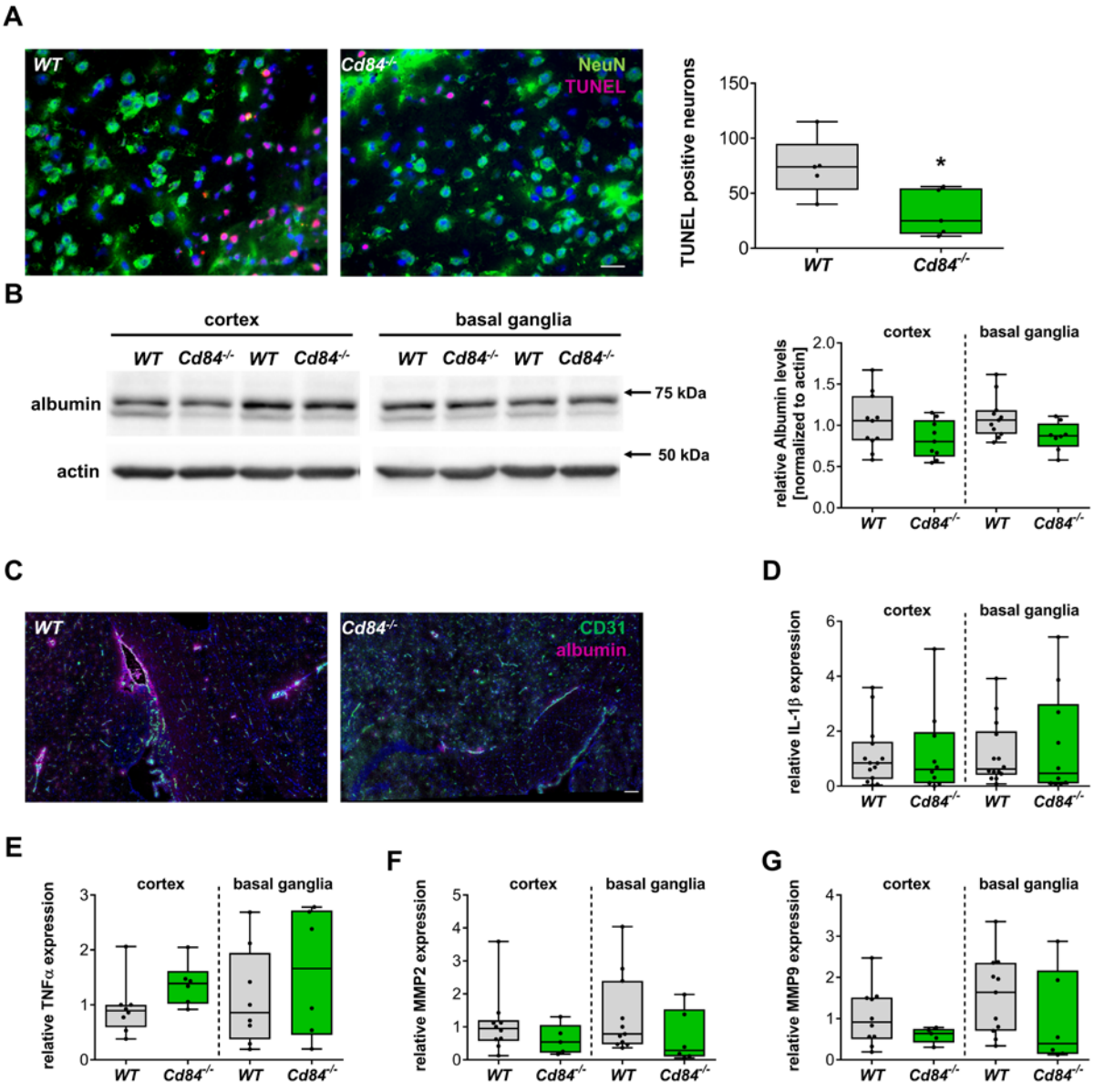
Online Figure II. Regional cerebral blood flow is not affected by CD84-deficiency, while infarct sizes following tMCAO are smaller in CD84-deficient male and female mice. (**A**) Regional cerebral blood flow (CBF) was measured with a Laser Doppler in *WT* and *Cd84*$^{-/-}$ mice (n = 4 per group) before (control), during tMCAO (ischemia) and directly after removal of the filament (reperfusion). (**B**) Sex-specific infarct volumes 24 h after tMCAO of *WT* (n = 10 male and 6 female mice) and *Cd84*$^{-/-}$ mice (n = 10 male and 8 female mice; p = 0.020 for the comparison of female mice). (**C**) Neuroscore of *WT* (n = 12 male and 6 female mice) and *Cd84*$^{-/-}$ mice (n = 13 male and 8 female mice; p = 0.0067 for the comparison of male mice) at 24 h after tMCAO. Statistical significances were determined using the Holm-Sidak correction on p-values obtained by Mann-Whitney tests. **P*<0.05 and ***P*<0.01.



Online Figure III. Role of CD84 in long-term functional outcome following stroke. (**A**) *WT* and *Cd84*$^{-/-}$ survival is depicted as Kaplan-Meier curve (n = 9-10 mice per group). (**B-F**) Neurological outcome over time in *WT* (gray boxes) and *Cd84*$^{-/-}$ mice (green boxes) as assessed by body swing (**B**), grip test (**C**), latency to move (**D**) and adhesive tape removal (**E,F**) after 30 min tMCAO (n = 7-10 mice per group). Statistical significances were determined using the Holm-Sidak correction obtained by Mann Whitney tests. **P*<0.05; exact n numbers and p-values are listed in Online Table V.

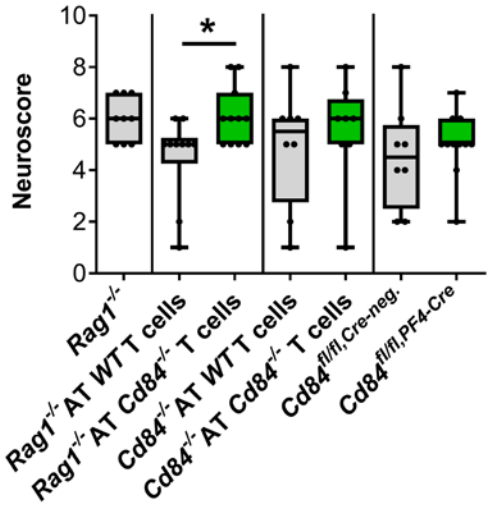


Online Figure IV. Immune cell recruitment to the brain on day 1 following 60 min tMCAO. Immune cell infiltration into the brain of *WT* and *Cd84*$^{-/-}$ mice was analyzed by flow cytometry. (**A**) Gating strategy for the flow cytometric analysis of immune cells. (**B**) Indicated immune cells per hemisphere (ipsi = ipsilateral, contra = contralateral hemisphere) 24 h after tMCAO of *WT* and *Cd84*$^{-/-}$ mice (n = 5 mice per group; no statistical significant differences were observed based on 2-way ANOVA).

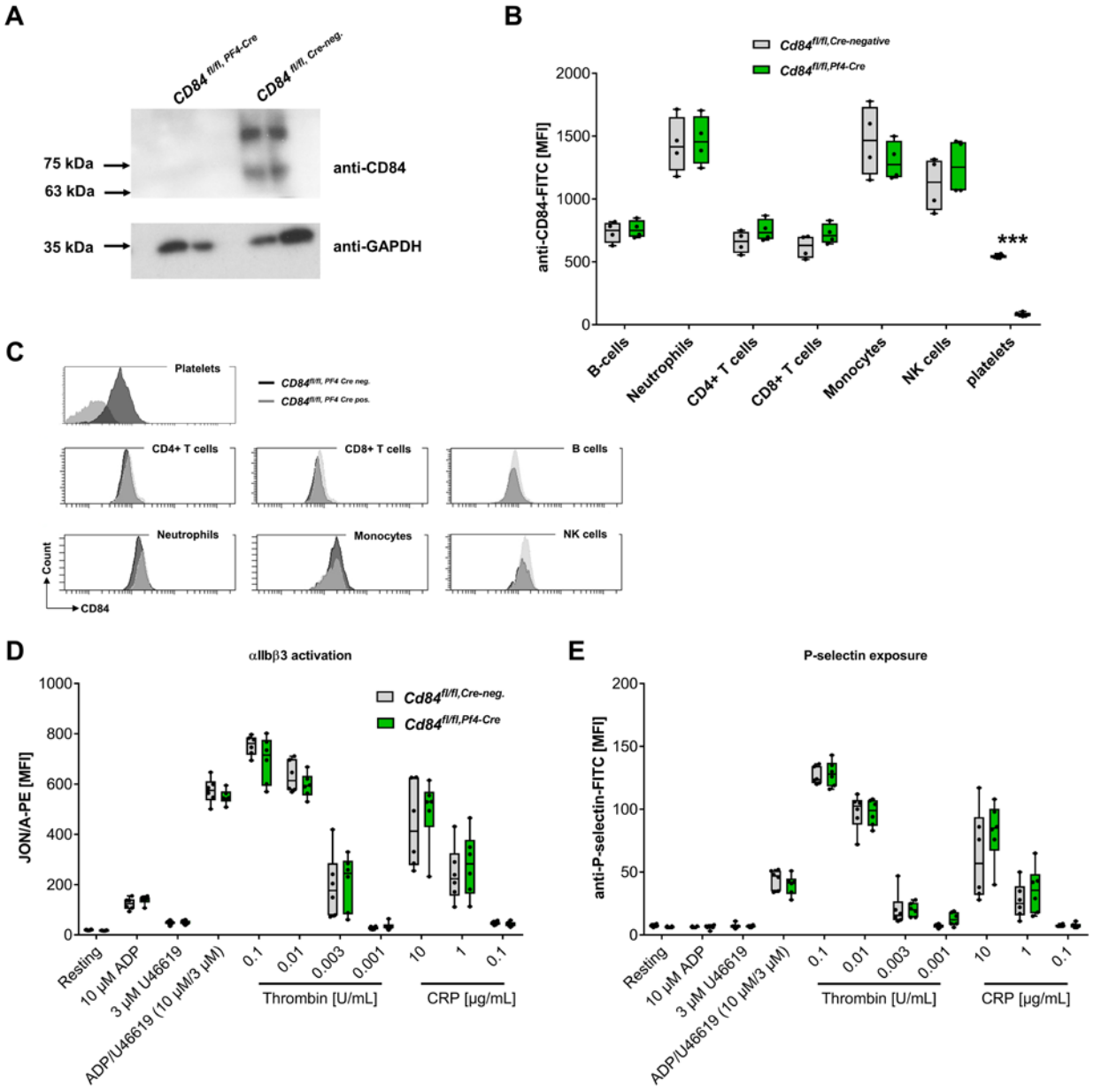


Online Figure V. Diminished neuronal apoptosis and blood brain barrier damage at day 1 after stroke in CD84-deficient mice. (**A**) Representative brain sections from a *WT* and a *Cd84*$^{-/-}$ mouse stained for the neuronal marker NeuN (green) and subjected to TUNEL assay (magenta) to visualize apoptosis. Quantification of dead neurons per optical field in the ischemic penumbra (n = 5 mice per group; p = 0.0317). Scale bar 25 µm. (**B**) Representative anti-albumin Western blot analysis and densitometric quantification of ipsilesional albumin protein levels in the basal ganglia (n = 11 for *WT* and n = 8 for *Cd84*$^{-/-}$ mice) as well as cortical regions (n = 11 for *WT* and n = 9 for *Cd84*$^{-/-}$ mice) after tMCAO. (**C**) Representative brain sections from a *WT* and a *Cd84*$^{-/-}$ mouse stained for vessels (anti-CD31, green) and albumin (magenta) to visualize leakage as indicator of blood brain barrier damage. Counter-stained with DAPI (blue), scale bar 100 µm. (**D**) Relative gene expression of interleukin (IL)-1β (n = 14 for *WT* and n = 10 for *Cd84*$^{-/-}$ mice), (**E**) tumor necrosis factor α (TNFα) (n = 8 for *WT* and n = 6 for *Cd84*$^{-/-}$ mice), (**F**) matrixmetalloproteinase 2 (MMP2) and (**G**) MMP9 in the ischemic basal ganglia (n = 11 for *WT* and n = 6 for *Cd84*$^{-/-}$ mice) and cortex (n = 10 for *WT* and n = 5 for *Cd84*$^{-/-}$ mice) at day 1 after tMCAO of *WT*

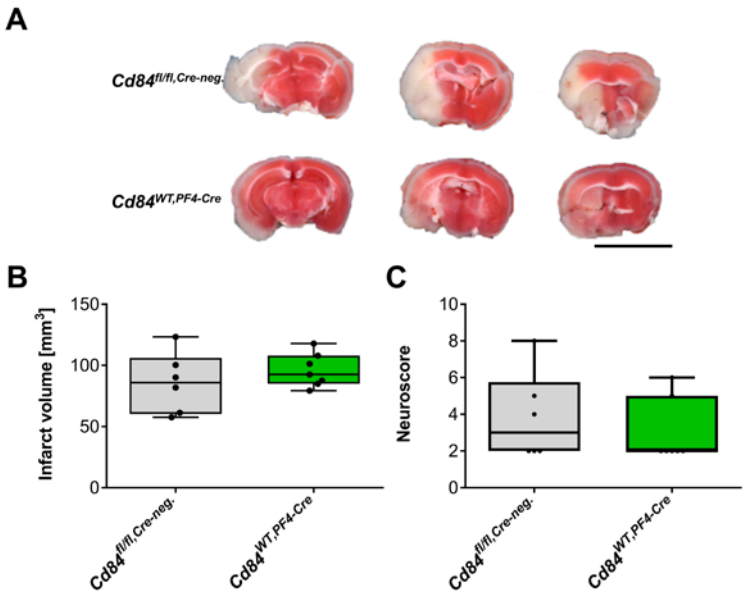
and *Cd84*$^{-/-}$ mice (n =5-6 mice per group). Statistical significances were analyzed by Mann-Whitney test: **P*<0.05.



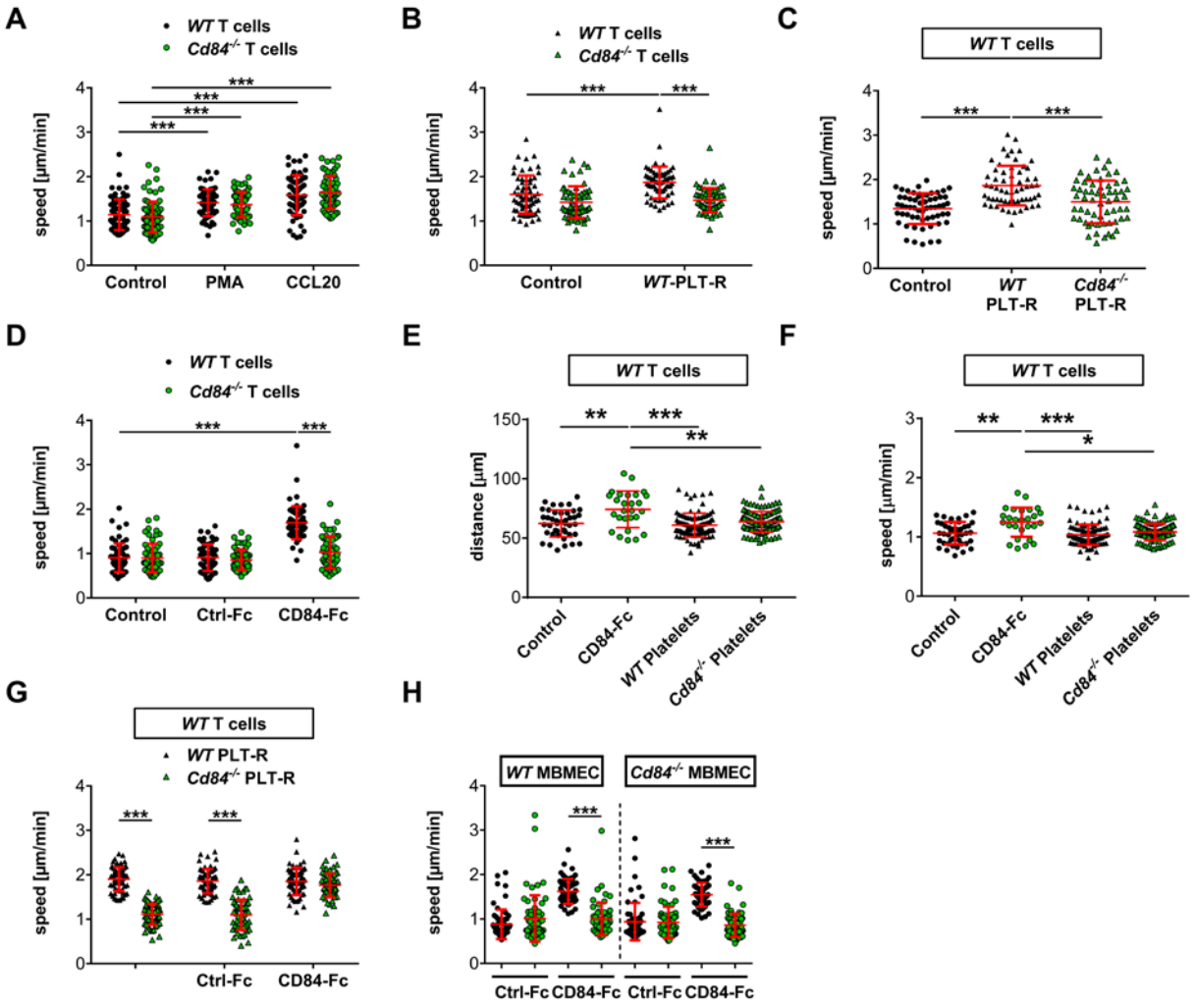
Online Figure VI. CD84-deficiency in CD4$^+$ T cells reduces cerebral damage. Neuroscore at day 1 after transient middle cerebral artery occlusion of *Rag1*$^{-/-}$ mice (n = 9), *Rag1*$^{-/-}$ mice with adoptive transfer of *WT* (n = 10) or *Cd84*$^{-/-}$ CD4$^+$ T cells (n = 11; p = 0.0225) at day 1 before surgery, *Cd84*$^{-/-}$ mice with adoptive transfer of *WT* (n = 8) or *Cd84*$^{-/-}$ CD4$^+$ T cells (n = 8) at day 1 before surgery, and mice specifically lacking the expression of CD84 on platelets (n = 11) compared to control mice (n = 8), at day 1 after tMCAO; AT = adoptive transfer. Statistical significances were analyzed by Mann-Whitney test. **P*<0.05



Online Figure VII. Normal activation responses in platelets from *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ mice. (**A**) Analysis of CD84 expression in platelets from two wild-type (*Cd84*$^{fl/fl,Cre\text{-}neg}$) and two *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ mice by Western blotting. Expression of GAPDH was used as loading control. (**B,C**) Flow cytometric analysis of CD84 expression of blood-derived immune cells (n = 4 per group; p>0.05) and platelets (n = 6 per group; p = $6.59*10^{-13}$) from wild-type (*Cd84*$^{fl/fl,Cre\text{-}neg}$) and *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ mice. (**B**) Results are presented as mean fluorescence intensities (MFI) and each dot represents one individual. (**C**) Representative histograms from *Cd84*$^{fl/fl,Cre\text{-}neg}$ and *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ derived cells. (**D,E**) Flow cytometric analysis of integrin αIIbβ3-activation (**D**) and degranulation-dependent P-selectin exposure (**E**) in response to the indicated agonists in wild-type and *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ platelets. Results are presented as mean fluorescence intensities (MFI) and are representative of 3 individual experiments with n = 6 mice per group. Statistical significances were determined using the Holm-Sidak correction on p-values obtained by Mann-Whitney tests. ***P<0.001; CRP: collagen-related peptide.



Online Figure VIII. Infarct sizes following tMCAO are comparable between *Cd84*$^{+/+;PF4Cre+ve}$ and *CD84*$^{fl/fl;\ PF4Cre-ve}$ mice. (**A**) Representative images of coronal sections stained with TTC 24 hours after tMCAO (60 min). Infarcted areas are shown in white; bar 1 cm. (**B**) Planimetric analysis was used to quantify the infarct volume of *Cd84*$^{+/+;\ PF4Cre+ve}$ and *CD84*$^{fl/fl;\ PF4Cre-ve}$ mice (n = 6-7 mice per group). (**C**) Neuroscore at 24 h after tMCAO (n = 6-7 mice per group). No statistical significant differences were observed using the Mann Whitney test.



Online Figure IX. Soluble CD84 promotes CD4$^+$ T cell migration. (**A**) Velocity of CD4-positive *WT* and *Cd84*$^{-/-}$ CD4$^+$ T cells treated with vehicle, phorbol-12-myristate-13-acetate (PMA), or C-C motif chemokine ligand 20 (CCL20). (**B**) *WT* and *Cd84*$^{-/-}$ CD4$^+$ T cell migration speed in response to stimulation with *WT* platelet releasate (PLT-R) compared to vehicle. (**C**) *WT* CD4$^+$ T cell migration speed in response to stimulation with *WT* or *Cd84*$^{-/-}$ PLT-R compared to control. (**D**) Velocity of CD4-positive *WT* and *Cd84*$^{-/-}$ CD4$^+$ T cells treated with vehicle, recombinant control-Fc or CD84-Fc protein. (**E**) Migration distance and (**F**) speed of *WT* CD4$^+$ T cells treated with vehicle (control), recombinant CD84-Fc protein or washed *WT* and *Cd84*$^{-/-}$ platelets. (**G**) *WT* CD4$^+$ T cell migration speed in response to stimulation with *WT* or *Cd84*$^{-/-}$ PLT-R in the presence of Control-Fc or recombinant CD84-Fc protein. (**H**) Velocity of CD4-positive *WT* and *Cd84*$^{-/-}$ CD4$^+$ T cells treated with GPVI-Fc or CD84-Fc on primary murine brain microvascular endothelial cells (MBMEC) of *WT* or *Cd84*$^{-/-}$ mice. Each dot represents the velocity of one CD4$^+$ T cell over 30 min (n = 27-120 cells per group of 3-4 independent experiments). Horizontal red lines correspond to the mean and standard deviation. Statistical significances analyzed by 1-way ANOVA with Bonferroni post hoc test or Kruskal-Wallis test with Dunn's multiple comparison test, the exact test, n-numbers and p-values are listed in Online Table VI; **P*<0.05; ***P*<0.01 and ****P*<0.001.

Online Table I. Exclusion criteria and dropout rates for transient middle cerebral artery occlusion (tMCAO) experiments.

Experimental group	Dropout rate	Sex (male/female)	Mortality rate
WT	4/26	16/10	6/26
Cd84$^{-/-}$	1/24	14/10	3/24
Rag1$^{-/-}$	0/9	7/2	0/9
Rag1$^{-/-}$ (AT of *WT* CD4^{+} T cells)	5/15	15/0	4/15
Rag1$^{-/-}$ (AT of *Cd84*$^{-/-}$ CD4^{+} T cells)	1/12	12/0	1/12
Cd84$^{-/-}$ (AT of *WT* CD4^{+} T cells)	0/8	8/0	0/8
Cd84$^{-/-}$ (AT of *Cd84*$^{-/-}$ CD4^{+} T cells)	0/8	8/0	0/8
Cd84$^{fl/fl,\ Cre\text{-}neg}$	2/10	10/0	0/10
Cd84$^{fl/fl,Pf4\text{-}Cre}$	0/11	11/0	0/11

AT = adoptive transfer

The following conditions excluded mice from analyses: (1) death within 24 hours after MCAO, (2) subarachnoidal hemorrhage or bleeding into the brain parenchyma (as macroscopically assessed during brain sampling, (3) Bederson score = 0 (24 hours after MCAO).

Assuming a reduction of infarct volume of 30% as functionally relevant and a standard deviation of 20% to the respective mean values, a group size of 8–10 was necessary to show this effect with a power of 0.8 and a probability of a type I error of <0.05 (calculated with GraphPad StatMate 2.00).

Online Table II. Cytokine production in CD4$^+$ T cells from naïve *Cd84*$^{-/-}$ and WT mice upon CD3/CD28 bead (48 h) stimulation in vitro.

	WT mice	*Cd84*$^{-/-}$ mice	*P*-value
Cytokine production (median (IQR))	n=4	n=4	
IFN-ɣ	22741 (12501-33926)	18070 (9150-28245)	0.83
IL-17	358.3 (265.3-756.9)	612.1 (519.9-1167)	0.17
IL-10	525.6 (346.9-1686)	607.6 (340.2-734.4)	>0.99

P-values were calculated by Mann-Whitney test.

Online Table III. Blood parameters and platelet glycoprotein expression of *Cd84*$^{fl/fl,Pf4\text{-}Cre}$ mice.

	Cd84$^{fl/fl,\ Cre\text{-}neg.}$ mice	*Cd84*$^{fl/fl,Pf4\text{-}Cre}$ mice	*P*-value
Blood parameters (median (IQR))	n=5	n=5	
Platelet count [10^3/mm³]	1166 (1137 1178)	1266 (1240-1381)	>0.9999
Mean platelet volume [µm³]	5.6 (5.6-5.7)	5.9 (5.7-5.9)	>0.9999
Hematocrit [%]	48.0 (47.9-48.0)	48.0 (46.7-48.4)	>0.9999
Hemoglobin [g/dl]	15.4 (15.4-15.6)	15.5 (15.1-15.5)	>0.9999
Red blood cell count [10^6/mm³]	10.3 (10.2-10.4)	10.4 (10.0-10.4)	>0.9999
White blood cell count [10^3/mm³]	9.2 (8.8-9.8)	8.7 (8.5-8.7)	>0.9999
Lymphocyte count [10^3/mm³]	7.4 (7.1-7.4)	6.9 (6.8-7.0)	>0.9999
Monocyte count [10^3/mm³]	0.2 (0.2-0.3)	0.2 (0.2-0.3)	>0.9999
Granulocyte count [10^3/mm³]	2.0 (1.6-2.1)	1.5 (1.5-1.5)	>0.9999
Eosinophil count [10^3/mm³]	0.0 (0.0-0.0)	0.1 (0.1-0.1)	>0.9999

Platelet glycoprotein expression (mean fluorescence intensity, median (IQR))	n=6	n=6	*P*-value
GPIbα	404 (377-424)	421 (414-430)	0.9653
GPVI	53 (52-53)	53 (52-55)	0.9145
Integrin α2	53 (53-53)	54 (54-55)	0.1221
CD84	39 (39-41)	8 (8-8)	0.0018
CD9	1039 (912-1093)	1105 (1084-1132)	0.9653
GPV	363 (347-371)	358 (347-364)	0.8056
GPIX	493 (487-504)	513 (510-518)	0.9653
Integrin αIIbβ3	460 (428-464)	449 (445-452)	0.9270
Integrin β3	180 (175-188)	166 (161-170)	0.3502
CLEC-2	179 (175-195)	205 (188-207)	0.9653
Integrin α5	33 (31-33)	30 (28-30)	0.8056

Statistical significance was determined using 2-way ANOVA with Sidak's multiple comparison test (blood parameters) and the Holm-Sidak method (glycoprotein expression), respectively.

Online Table IV. Characterization of the SICFAIL study population (n = 98), stratified for NIHSS score at day three of hospitalization.

	NIHSS < 5	NIHSS ≥ 5	p-value†
	n = 79	n = 19	
Demographics			
Age, years, median (IQR)	65 (52, 74)	77 (71, 82)	0.0001
Male, n (%)	52 (65.8)	13 (68.4)	0.83
Comorbidities			
Previous Stroke or TIA, n (%)	22 (31.9)	3 (18.8)	0.38
Coronary Heart Disease, n (%) *	10 (14.5)	4 (25.0)	0.45
Hypertension, n (%)	50 (68.5)	11 (78.6)	0.54
Type 2 Diabetes mellitus, n (%)	13 (18.1)	5 (27.8)	0.34
Atrial fibrillation, n (%)	7 (11.1)	4 (25.0)	0.22
Index Event			
NIHSS on admission, median (IQR)	3 (2-5)	8 (4-14)	< 0.0001
Thrombolysis, n (%)	20 (27.0)	7 (38.9)	0.32
Time from symptom onset to blood draw, hours, median (IQR)	68.1 (48.0-93.3)	84.1 (62.3-104.9)	0.19
Anti-CD84 mean fluorescence intensity, median (IQR)	23.1 (21.2-25.1)	23.6 (22.6-25.5)	0.23

* defined as history in myocardial infarction and/or angina pectoris

NIHSS National Institute of Health Stroke Scale; MFI mean fluorescence intensity

P-values were calculated by non-parametric Mann-Whitney test for continuous variables and by Chi Square test or Fisher's exact test for categorical variables.

Online Table V. P-values and n numbers of long-term functional outcome following stroke data (Figure 2 and Online Figure III).

		d (post tMCAO)	**-1**	**1**	**2**	**3**	**4**	**5**	**7**
		n (WT)	10	10	9	9	8	7	7
Fig.	**Experiment**	**n (KO)**	10	9	9	9	9	9	9
2a	mNSS	**p-values**	-	0.0111	0.0013	0.0013	0.0565	0.0111	0.1721
2b	Corner Test	**p-values**	-	0.0017	0.0109	0.0109	4.58E-05	4.75E-04	0.0109
2c	Bederson Score	**p-values**	-	0.1006	0.1006	0.0161	0.1006	0.0439	0.0439
OIIIB	Body Swing Test	**p-values**	-	0.1757	0.0180	0.1689	0.1317	0.0663	0.1317
OIIIC	Grip Test	**p-values**	0.6387	0.5948	0.2066	0.4404	0.9779	0.9779	0.5948
OIIID	Latency to move	**p-values**	0.7920	0.0155	0.7920	0.1022	0.7920	0.6695	0.7920
OIIIE	Adhesive Tape (left paw)	**p-values**	0.7511	0.3352	0.1014	0.0053	0.3352	0.8717	0.6524
OIIIF	Adhesive Tape (right paw)	**p-values**	0.9190	0.3697	0.7572	0.7572	0.0719	0.0437	0.1317

Statistical significance was determined using the Holm-Sidak method.

Online Table VI. P-values and n numbers of T cell migration assays (Figure 5 and Online Figure IX).

Figure 4A/OIX A		*WT* (control)	*Cd84*$^{-/-}$ (control)	*WT* (PMA)	*Cd84*$^{-/-}$ (PMA)	*WT* (CCL20)	*Cd84*$^{-/-}$ (CCL20)
n		80	80	60	60	59	60
p-value (vs. control of same genotype)	distance (Fig. 4A)	-	-	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	speed (Fig. OIX A)	-	-	0.0001	< 0.0001	< 0.0001	< 0.0001
p-value (vs. *WT* with same stimulus)	distance (Fig. 4A)	-	> 0.9999	-	> 0.9999	-	> 0.9999
	speed (Fig. OIX A)	-	> 0.9999	-	> 0.9999	-	> 0.9999
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test					

Figure 4B/OIX B		*WT* (control)	*Cd84*$^{-/-}$ (control)	*WT* (*WT* PLT-R)	*Cd84*$^{-/-}$ (*WT* PLT-R)
n		60	60	59	59
p-value (vs. control of same genotype)	distance (Fig. 4B)	-	-	< 0.0001	> 0.9999
	speed (Fig. OIX B)	-	-	0.0002	> 0.9999
p-value (vs. *WT* with same stimulus)	distance (Fig. 4B)	-	> 0.9999	-	< 0.0001
	speed (Fig. OIX B)	-	0.083	-	< 0.0001
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test			

Figure 4C/OIX C		*WT* T cells stimulated with:		
		Control	*WT* PLT-R	*Cd84*$^{-/-}$ PLT-R
n		60	60	59
p-value (vs. control of same genotype)	distance (Fig. 4C)	-	< 0.0001	0.5778
	speed (Fig. OIX C)	-	< 0.0001	0.156
p-value (vs. *WT* with same stimulus)	distance (Fig. 4C)	-	-	< 0.0001
	speed (Fig. OIX C)	-	-	< 0.0001
Signifcance assessed by		One-way ANOVA with Bonferroni multiple comparison		

Figure 4D/OIX D		*WT* (control)	*Cd84*$^{-/-}$ (control)	*WT* (Ctr-Fc)	*Cd84*$^{-/-}$ (Ctr-Fc)	*WT* (CD84-Fc)	*Cd84*$^{-/-}$ (CD84-Fc)
n		60	60	60	60	60	60
p-value (vs. control of same genotype)	distance (Fig. 4D)	-	-	> 0.9999	> 0.9999	< 0.0001	> 0.9999
	speed (Fig. OIX D)	-	-	> 0.9999	> 0.9999	< 0.0001	> 0.9999
p-value (vs. *WT* with same stimulus)	distance (Fig. 4D)	-	> 0.9999	-	> 0.9999	-	< 0.0001
	speed (Fig. OIX D)	-	> 0.9999	-	> 0.9999	-	< 0.0001
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test					

Figure 4E/OIX G		*WT* T cells stimulated with:					
		WT PLT-R	*Cd84*$^{-/-}$ PLT-R	*WT* PLT-R + Ctr-Fc	*Cd84*$^{-/-}$ PLT-R + Ctr-Fc	*WT* PLT-R + CD84-Fc	*Cd84*$^{-/-}$ PLT-R+ CD84-Fc
n		60	60	60	60	60	60
p-value (vs. control of same genotype)	distance (Fig. 4E)	-	-	> 0.9999	> 0.9999	> 0.9999	< 0.0001
	speed (Fig. OIX G)	-	-	> 0.9999	> 0.9999	> 0.9999	< 0.0001
p-value (vs. *WT* with same stimulus)	distance (Fig. 4E)	-	< 0.0001	-	< 0.0001	-	> 0.9999
	speed (Fig. OIX G)	-	< 0.0001	-	< 0.0001	-	> 0.9999
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test					

Figure 4F/OIXH		Experiment on *WT* MBMEC				Experiment on *Cd84*$^{-/-}$ MBMEC			
		WT (Ctr-Fc)	*Cd84*$^{-/-}$ (Ctr-Fc)	*WT* (CD84-Fc)	*Cd84*$^{-/-}$ (CD84-Fc)	*WT* (Ctr-Fc)	*Cd84*$^{-/-}$ (Ctr-Fc)	*WT* (CD84-Fc)	*Cd84*$^{-/-}$ (CD84-Fc)
n		60	60	60	60	60	60	60	60
p-value (vs. control of same genotype)	distance (Fig. 4F)	-	-	< 0.0001	> 0.9999	-	-	< 0.0001	> 0.9999
	speed (Fig. OIX H)	-	-	< 0.0001	> 0.9999	-	-	< 0.0001	> 0.9999
p-value (vs. *WT* with same stimulus)	distance (Fig. 4F)	-	0.182	-	< 0.0001	-	> 0.9999	-	< 0.0001
	speed (Fig. OIX H)	-	> 0.9999	-	< 0.0001	-	> 0.9999	-	< 0.0001
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test							

Figure OIXE/OIXF		Distance (Figure OIXE)				Speed (Figure OIXF)			
		WT T cells stimulated with:				*WT* T cells stimulated with:			
		Ctr-Fc	CD84-Fc	*WT* platelets	*Cd84*$^{-/-}$ platelets	Ctr-Fc	CD84-Fc	*WT* platelets	*Cd84*$^{-/-}$ platelets
n		43	27	95	120	43	27	95	120
p-value compared to	Ctr-Fc	-	0.0098	> 0.9999	> 0.9999	-	0.0089	> 0.9999	> 0.9999
	CD84-Fc	0.0098	-	< 0.0001	0.0106	0.0089	-	< 0.0001	0.0108
	WT platelets	> 0.9999	< 0.0001	-	0.0835	> 0.9999	< 0.0001	-	0.0108
	Cd84$^{-/-}$ platelets	> 0.9999	0.0106	0.0835	-	> 0.9999	0.0108	0.0108	-
Signifcance assessed by		Kruskal-Wallis test with Dunn´s multiple comparison test							