SUPPLEMENTARY INFORMATION

RHBDL2 links cerebral infarction to endothelial dysfunction in pneumococcal meningitis

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SUPPLEMENTARY FIGURES

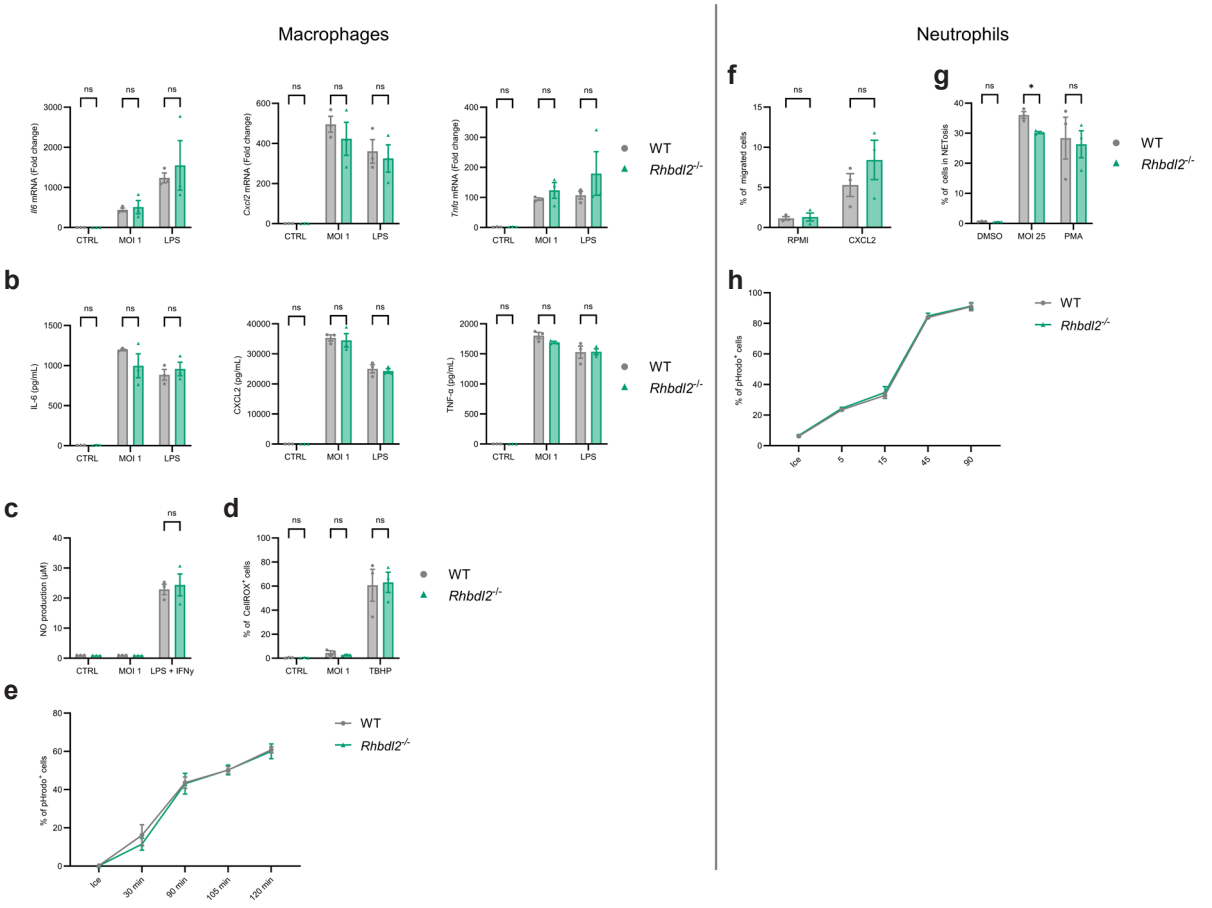
Figure S1. Functional evaluation of RHBDL2 knockout in murine macrophages and neutrophils.
BMDMs and neutrophils were isolated from *Rhbdl2*$^{-/-}$ and control mice (WT) and left untreated (CTRL) or stimulated with LPS (10 ng/mL) or *S. pneumoniae* (MOI 1) for 24 hours. (a) *Cxcl2, Il-6* and *Tnf* mRNA expression in BMDMs. (b) CXCL2, IL-6 and TNF-α concentrations and (c) nitric oxide production in BMDM supernatants. (d) ROS production was measured using CellROX in BMDMs. BMDMs were stimulated with either PBS (CTRL), *S. pneumoniae* (MOI 1) or 0.4 mM TBHP for one hour. (e) Phagocytosis of pHrodo labelled *E. coli* bioparticles after 60 minutes of incubation in BMDMs. Controls were incubated without bioparticles or with bioparticles in the presence of cytochalasin D, an inhibitor of phagocytosis. (f) Migration of neutrophils through transwells in response to CXCL2 or RPMI (control) was measured after 4 hours. (g) Neutrophil NET production was determined upon stimulation with *S. pneumoniae* (MOI 25), PMA or DMSO (control), using SYTOX Green dye. Data are presented as median ± SEM. N = 3 mice per genotype for all analyses. Group differences were calculated using a t-test. Ns, non-significant; *, p-value < 0.05. BMDMs, bone marrow-derived macrophages; CXCL2, chemokine (C-X-C motif) ligand 2; DMSO, dimethylsulfoxide; IL, interleukin; TNF, Tumor Necrosis Factor; LPS, lipopolysaccharide; MOI, multiplicity of infection; NET, neutrophil extracellular traps; PMA, Phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute Medium; TBHP, tert-butylhydroperoxide

Figure S2. BCAM expression in the murine brain
Rhbdl2$^{-/-}$ and wild-type (WT) mice were intracisternally injected with *S. pneumoniae* (infected) or PBS (control) and sacrificed at 20 hpi. (a, b) Representative images of histological brain slides stained with anti-BCAM and haematoxylin. Images are taken at a magnification of 200x. N = 12 (infected) or 6 (control) mice per genotype. BCAM, basal cell adhesion molecule; Hpi, hours post infection; PBS, phosphate buffered saline.

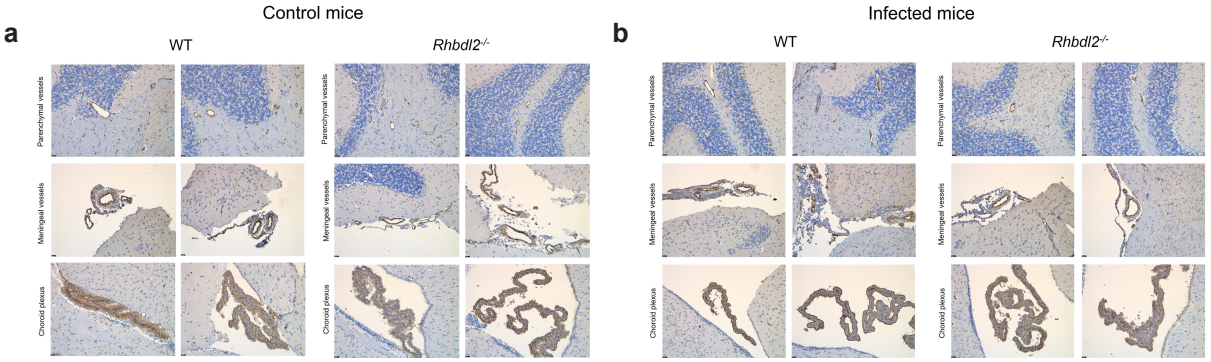
Figure S3. VE-cadherin expression in the murine brain
Rhbdl2$^{-/-}$ and wild-type (WT) mice were intracisternally injected with *S. pneumoniae* (infected) or PBS (control) and sacrificed at 20 hpi. Protein expression of VE-cadherin in the brain was determined by western blotting. Protein expression was normalized against transferrin. N = 7 mice per genoypte. PBS, phosphate buffered saline. VE-cadherin, vascular endothelial cadherin.

Figure S4. Validation of Rhbdl2 knockout in mice
To confirm knockout of *Rhbdl2*, *Rhbdl2* mRNA expression was measured in the brain and kidney of *Rhbdl2*$^{-/-}$ and wild-type (WT) by RT-qPCR. N = 3 mice per genotype. mRNA, messenger ribonucleic acid; RT-qPCR, Reverse Transcription-quantitative Polymerase Chain Reaction.

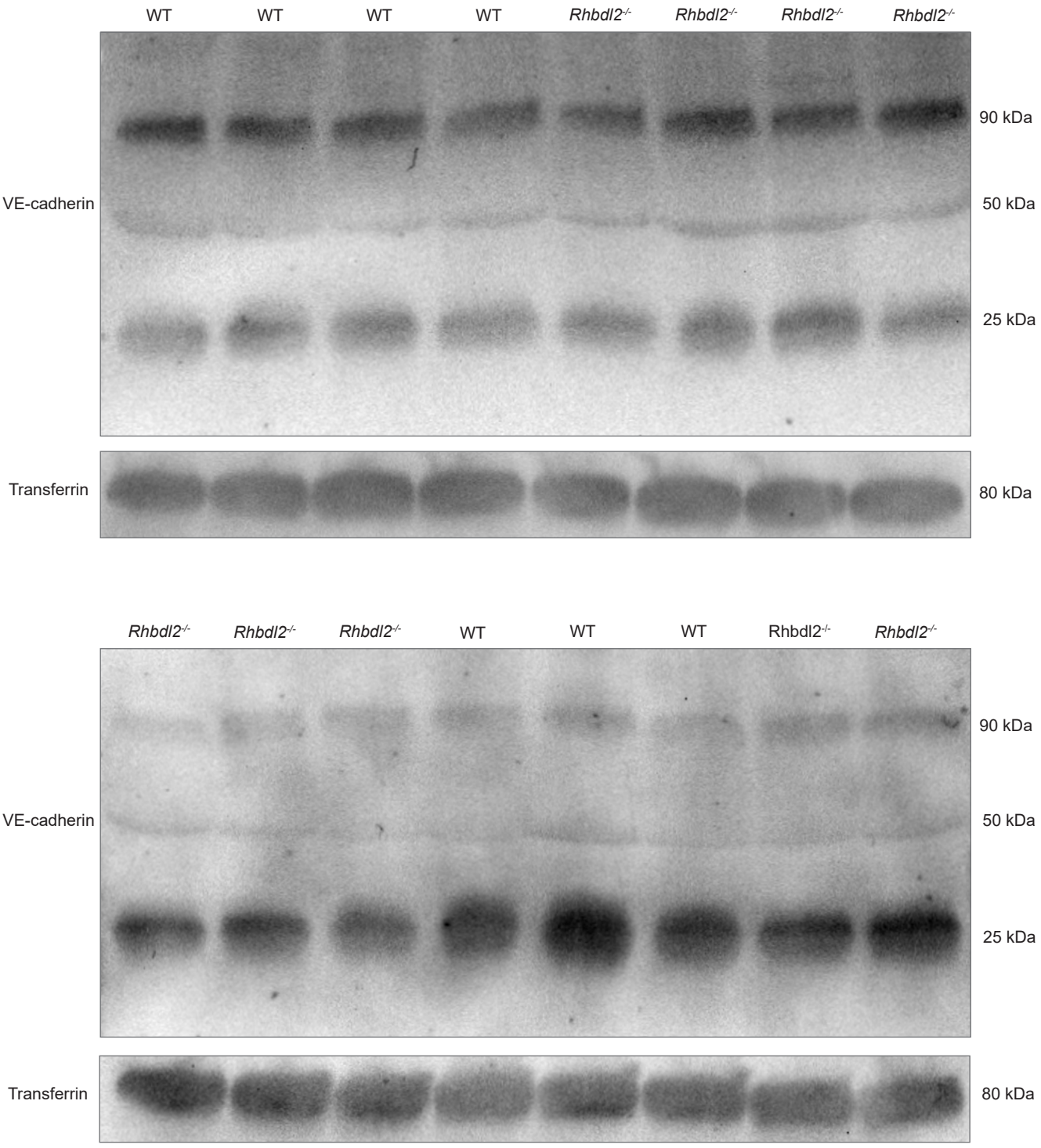
Supplementary Figure 1 a



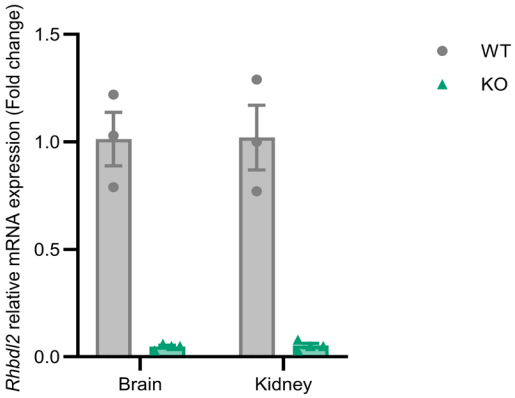
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



SUPPLEMENTARY TABLES

Supplementary table 1. Multivariate logistic regression of rs143464422 genotype for infarction in pneumococcal meningitis

	No infarction N = 642	**Infarction** N = 102	**Multivariable odds ratio for infarction**	**p value of multivariable analysis**
rs143464422 genotype				
G	586/642 (91%)	74/102 (73%)	*Reference*	
A	56/642 (9%)	28/102 (27%)	4.00 (2.35–6.69)	<0.001
Age – years[a]	60 (48-68)	63 (53-70)	1.01 (1.00-1.03)	0.067
Female sex	333/642 (52%)	53/102 (52%)	0.95 (0.61-1.46)	0.8
Immunocompromised[b]	154/640 (24%)	29/102 (28%)	1.26 (0.77-2.02)	0.3

Data presented as n/N (%), median (IQR) or odds ratio (95% confidence interval). The dominant genetic model includes the GG genotype as the reference group (G), and the AG/GA and AA genotypes as the alternative group (A). Age is known for all patients. [b]Immunocompromised is defined as active cancer, diabetes, alcoholism, immunosuppressive treatment, splenectomy or HIV.

Supplementary table 2: Baseline characteristics of pneumococcal meningitis patients with CSF measurements

	N = 192
Age – years[a]	61 (47-69)
Female sex	98/192 (51%)
Immunocompromised state[b]	54/192 (28%)
Symptoms on admission	
Headache	137/166 (83%)
Temperature - °C[c]	39.0 (38.2-39.7)
GCS score[d]	10 (9-13)
Blood chemical tests	
Leukocytes - cells/mL[e]	17.5 (12.0-23.3)
Thrombocytes – cells/mL[f]	206 (156-256)
C-reactive protein - mg/L[g]	197 (88-299)
Indices or CSF inflammation	
Leukocytes - cells/mm3[h]	2,978 (598-7,733)
CSF protein - g/L[i]	3.9 (2.4-6.0)
CSF:blood glucose ratio[j]	0.03 (0.01-0.26)
Dexamethasone treatment	184/192 (96%)
Outcome	
Mortality	15/192 (8%)
Unfavorable outcome	70/192 (36%)

In a subset of genotyped patients CSF measurements were performed of EGF, fibrinogen, PAI-1, PAI-2 and sVCAM-1. Data presented as n/N (%) or median (IQR). Abbreviations: CSF, cerebrospinal fluid; EGF, epidermal growth factor; GCS, Glasgow Coma Scale; PAI, plasminogen activator inhibitor; sVCAM, soluble vascular cell adhesion protein. [a]Age is known for all patients. [b]Immunocompromised state is defined as active cancer, diabetes, alcoholism, immunosuppressive treatment, splenectomy or HIV. [c]Temperature is known in 191 episodes. [d]GCS is known for all patients. [e]Leukocyte count in blood is known for all patients. [f]Thrombocyte count in blood is known in 188 episodes. [g]C-reactive protein in blood is known in 184 episodes. [h]Leukocyte count in CSF is known in 186 episodes. [i]Protein concentration in CSF is known in 186 episodes. [j]CSF:blood glucose ratio is known in 182 episodes.

Supplementary table 3: Baseline characteristics of pneumococcal meningitis patients with CSF thrombomodulin measurements

	N = 183
Age – years[a]	62 (50-68)
Female sex	91/183 (50%)
Immunocompromised state[b]	38/183 (21%)
Symptoms on admission	
Headache	130/160 (81%)
Temperature - °C[c]	39.0 (37.8-39.7)
GCS score[d]	10 (9-13)
Blood chemical tests	
Leukocytes - cells/mL[e]	16.4 (12.6-23.8)
Thrombocytes – cells/mL[f]	206 (152-256)
C-reactive protein - mg/L[g]	202 (86-316)
Indices or CSF inflammation	
Leukocytes - cells/mm3[h]	2,280 (401-6,830)
CSF protein - g/L[i]	4.2 (2.7-6.0)
CSF:blood glucose ratio[j]	0.02 (0.01-0.21)
Dexamethasone treatment	169/176 (96%)
Outcome	
Mortality	12/183 (7%)
Unfavorable outcome	63/183 (34%)

Data presented as n/N (%) or median (IQR). Abbreviations: CSF, cerebrospinal fluid; GCS, Glasgow Coma Scale. [a]Age is known for all patients. [b]Immunocompromised state is defined as active cancer, diabetes, alcoholism, immunosuppressive treatment, splenectomy or HIV. [c]Temperature is known in 181 episodes. [d]GCS is known for all patients. [e]Leukocyte count in blood is known for all patients. [f]Thrombocyte count in blood is known in 174 episodes. [g]C-reactive protein in blood is known in 179 episodes. [h]Leukocyte count in CSF is known in 179 episodes. [i]Protein concentration in CSF is known in 177 episodes. [j]CSF:blood glucose ratio is known in 174 episodes.

Supplementary Table 4. Clinical severity score for murine pneumococcal meningitis

Code	Item	Score
	Appearance	
	Weight loss	
1	< 5% weight loss	1
2	5-10% weight loss	2
3	11-15% weight loss	3
4	16-20% weight loss	4
5	≥20% weight loss	HEP
	Posture	
1	Slightly hunched back	1
2	Severe hunched back	2
	Coat	
1	Diminished/lack grooming	1
2	Piloerection	1
3	Combination 1+2	2
	Eyes	
1	Discharge from the eyes	1
2	Closed eyelids	1
3	Protruding eyes	1
4	Combination 1+2	2
5	Combination 1+3	2
	Behaviour	
	Activity	
1	Diminished activity	2
2	Inactive	3
3	Increased activity / aggressive behaviour	1
	Condition	
1	Within 5 sec to right when placed on back	2
2	Within 30 sec to right when placed on back	4
3	Inability to right after placing on back	HEP
4	Coma	HEP
	Body function	
	Respiration	
1	Laboured breathing	2
2	Irregular breathing,	2
3	Combination 1+2	4
	Procedure-specific indicators	
	Neurologic score	
1	Paresis	2
2	Coordination problem (including circling)	2
3	(Partial) seizure	2
4	Combination 1+2	4
5	Combination 1+3	4
6	Combination 2+3	4
7	Combination 1+2+3	6
8	Paralysis	HEP
9	Seizure > 5 minutes	HEP
10	≥ 2 seizures in 15 min	HEP

HEP, humane endpoint

Supplementary Table 5. Histopathological scoring method of brain tissue in bacterial meningitis mouse model.

Main category	Subcategory	Score			
		0	1	2	3
Meningeal infiltration		Absent	Focal mild infiltration	Multifocal mild or focal severe infiltration	Multifocal severe infiltration
Parenchymal infiltration		Absent	Focal mild infiltration	Multifocal mild or focal severe infiltration	Multifocal severe infiltration
Vascular inflammation	Large meningeal artery inflammation Small parenchymal vessel inflammation	Absent	Focal mild subendothelial infiltration /reactive changes	Multifocal mild subendothelial infiltration /reactive changes or focal severe vascular wall infiltration with obstruction and/or destruction of vessels	Multifocal severe vascular wall infiltration with obstruction and/or destruction of vessels
Ventriculitis		Absent	A few inflammatory cells in the ventricle	Groups of inflammatory cells in the ventricle with/without ependymal infiltration	Extension of inflammatory cells into the periventricular tissue
Hemorrhage		Absent	Focal small damage	Multifocal small or focal large damage	Multifocal large damages
Thrombosis		Absent	Focal mild with partial obstruction of vascular lumen	Multifocal mild with partial obstruction of vascular lumen or focal severe with complete obstruction of vascular lumen and destruction of vessel wall	Multifocal severe with complete obstruction of vascular lumen and destruction of vessel wall
Abscess		Absent	Focal small damage	Multifocal small or focal large damage	Multifocal large damages

Supplementary Table 6. List of primers

Gene	Organism	5' forward primer	3' reverse primer
Rhbdl2	*Mus musculus*	TTGGAATCGTCAGACTACTCGT	AAGGACACCGGAGACCCATT
B-actin	*Mus musculus*	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCA
Rpl13a	*Mus musculus*	GGGCAGGTTCTGGTATTGGAT	GGCTCGGAAATGGTAGGGG
Il6	*Mus musculus*	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACTCTTT
Tnfα	*Mus musculus*	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Cxcl2	*Mus musculus*	AAGTTTGCCTTGACCTGAAG	ATCAGGTACGATCCAGGCTTC
Il1β	*Mus musculus*	GCCACCTTTTGACAGTGATGA	AAGCTGGATGCTCTCATCAGG