

RHBDL2 links cerebral infarction to endothelial dysfunction in pneumococcal meningitis

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Abstract

Cerebral infarction is a frequent and devastating complication of pneumococcal meningitis, yet the underlying host mechanisms remain poorly defined. We performed a genome-wide association study in adults with pneumococcal meningitis to identify genetic variants associated with cerebral infarction, followed by cerebrospinal fluid biomarker analyses and functional validation in a *Rhbdl2*-deficient mouse model. We identified a genome-wide association locus at chromosome 1 mapping to RHBDL2, encoding for an endothelial intramembrane serine protease. Carriers of the risk allele exhibited increased markers of endothelial activation and coagulation in cerebrospinal fluid. In experimental pneumococcal meningitis, *Rhbdl2* deficiency resulted in reduced bacterial outgrowth in the brain and spleen, attenuated cerebral inflammation, and altered endothelial function, marked by loss of VE-cadherin. Together, these findings identify the RHBDL2 locus as a genetic determinant of cerebrovascular complications in pneumococcal meningitis and suggest that RHBDL2-mediated endothelial remodeling represents a key pathway linking host genetic susceptibility to vascular injury in severe infection.

INTRODUCTION

Pneumococcal meningitis, caused by *Streptococcus pneumoniae*, is a life-threatening infection of the central nervous system, with mortality rates of approximately 20% and unfavorable outcome reported in up to half of affected patients despite advances in vaccination and adjunctive anti-inflammatory therapy¹⁻⁵. Cerebrovascular complications, particularly cerebral infarction, occur in about one quarter of patients and represents a major determinant of mortality and long-term morbidity⁵⁻⁷. Although vascular involvement is a hallmark of severe disease, the host mechanisms that predispose patients to cerebral infarction during meningitis remain incompletely understood⁸.

Clinical risk factors associated with cerebral infarction include advanced age, impaired level of consciousness, lower cerebrospinal fluid (CSF) white cell counts, and elevated systemic inflammatory markers⁹. Beyond these clinical features, accumulating evidence suggests that the host genetic variation contributes to the development of cerebrovascular complications. Previous candidate gene studies have implicated variation in coagulation and fibrinolysis pathways, including *SERPINE1* and *PROZ*, in the risk of cerebral infarctions and adverse outcome during bacterial meningitis^{10,11}. However, these studies were hypothesis driven, and unbiased genome-wide approaches to identify genetic determinants of vascular injury in pneumococcal meningitis are lacking.

The cerebral vasculature plays a central role in the pathophysiology of meningitis⁸. Endothelial cells regulate leukocyte trafficking, coagulation, and barrier integrity, and are dynamically remodeled during infection and inflammation. Disruption or maladaptive remodeling of endothelial junctions has been implicated in blood-brain barrier dysfunction, vascular inflammation, and ischemic injury in infectious and inflammatory diseases⁸. Nevertheless, the specific endothelial pathways that modulate vascular remodeling and susceptibility to cerebral infarction in bacterial meningitis remain poorly defined.

In this study, we performed a genome-wide association study in adults with pneumococcal meningitis to identify genetic variants associated with cerebral infarction. We identified a genome-wide significant locus encompassing *RHBDL2*, encoding a rhomboid intramembrane protease expressed in endothelial cells. By integrating human genetic data with CSF biomarker analyses and functional validation in a *Rhbdl2*-deficient mouse model of pneumococcal meningitis, we demonstrate that RHBDL2 modulates endothelial junctional remodeling, vascular inflammation, and disease outcome. These findings identify a previously unrecognized endothelial pathway linking host genetics to cerebrovascular complications in severe infection.

RESULTS

A genetic locus in *RHBDL2* confers risk of cerebral infarction complicating pneumococcal meningitis

We analyzed genome-wide genotype data from 744 adults with pneumococcal meningitis, of whom 102 (14%) developed cerebral infarction during disease course. Patients with cerebral infarction had a median age of 63 years (interquartile range [IQR] 53-70 years) versus a median age of 60 (IQR 48-68) for patients without cerebral infarction; a median Glasgow Coma Scale score at admission of 10 (IQR 9- 12) versus 11 (IQR 9- 13); were immunocompromised in 29 of 102 (28%) versus 152 of 640 (24%) of patients; and were treated with dexamethasone in 87 of 96 (91%) versus 591 of 633 (93%) (Table 1). Patients with cerebral infarction had markedly worse outcomes, including higher rates of unfavorable outcome (68% vs 25%) and mortality (23% vs 5%) compared to patients without infarction (Table 1).

Following imputation and quality control, 6.8 million markers were assessed for an association with cerebral infarctions. A single locus on chromosome 1 reached genome-wide significance ($p < 5.0 \times 10^{-8}$), with the strongest association at SNP rs143464422 (chr1:39373167 G>A; $p < 3.9 \times 10^{-9}$; Fig. 1a). This variant maps to an intronic region of *RHBDL2*, encoding the intramembrane serine protease rhomboid-like protein 2 (Fig. 1b). In cases, 74 of 102 (73%) were homozygous for the G allele, 26 (25%) were heterozygous and 2 (2%) were homozygous for the A allele. In controls, 586 of 642 (92%) were homozygous for the G allele, 53 (8%) were heterozygous and 3 (0.5%) were homozygous for the A allele (Table 2). To assess whether the association between rs143464422 and cerebral infarction was independent of established baseline clinical risk factors, we performed multivariable logistic regression adjusting for age, sex, and immunocompromised status. The association between the rs143464422 risk allele and cerebral infarction remained robust after adjustment (adjusted odds ratio 3.37, 95% CI 2.08–5.40, $p = 5.27 \times 10^{-7}$), indicating that the genetic effect was independent of baseline clinical predictors (Supplementary Table 1). Carriers of the risk allele experienced more frequent respiratory failure (33% vs 21%; $p = 0.021$), paresis (13% vs 5%; $p = 0.020$), and higher mortality than non-carriers (14% vs 7%; $p = 0.023$, Table 2).

***RHBDL2* risk allele associates with altered coagulation and endothelial activation in cerebrospinal fluid**

To explore biological pathways linked to the rs143464422 genotype, we measured coagulation and inflammatory markers in CSF from 192 patients, including 25 (13%) carriers of the risk allele (Supplementary Table 2). Risk allele carriers exhibited higher CSF concentrations of plasminogen activator inhibitor-2 (PAI-2), an inhibitor of tissue plasminogen activator, and elevated soluble VCAM-1, a marker of endothelial activation (Fig. 2a). In contrast, other coagulation markers, such as fibrinogen and PAI-1, did not differ between genotypes. Thrombomodulin, measured in an independent cohort of 183 patients (Supplementary Table 2), was also similar between genotypes.

Because RHBDL2 protein itself was not detectable in the CSF, we assessed levels of epidermal growth factor (EGF), a known RHBDL2 substrate. CSF EGF concentrations were markedly reduced in risk allele carriers (Fig. 2b), suggesting reduced RHBDL2 proteolytic activity associated with the infarction risk genotype.

***Rhbdl2* modulates bacterial burden and neuroinflammation in experimental meningitis**

To investigate the functional role of *Rhbdl2*, we compared disease course in *Rhbdl2* knockout mice (*Rhbdl2*^{-/-}) and wild-type mice subjected to experimental pneumococcal meningitis (Fig. 3a). Although overall survival and clinical scores were similar (Fig. 3b), *Rhbdl2*^{-/-} mice showed reduced bacterial outgrowth in the brain and spleen at 20 hours post-infection (Fig. 3c). This was accompanied by reduced cerebral expression of *Il1β* and *Cxcl2*, whereas *Il6* expression was unchanged (Fig. 3d,e). Histopathological analysis revealed reduced parenchymal immune cell infiltration with lower overall pathology scores in *Rhbdl2*^{-/-} mice (Fig. 3f,g). The number and size of cerebral hemorrhages did not differ between the two groups (Fig. 3h) and infarction was not observed in either genotype, which is consistent with known limitations of this model¹².

***Rhbdl2* regulates endothelial activation and coagulation independently of innate immune cell function**

Ex vivo stimulation of bone marrow-derived macrophages and neutrophils revealed no genotype-dependent differences in cytokine production or phagocytosis, indicating that *Rhbdl2* does not directly modulate innate immune cell effector function (Supplementary Fig. 1). In the brain, vascular permeability assessed by IgG extravasation was reduced in *Rhbdl2*^{-/-} mice, an effect that is likely attributable to lower bacterial burden (Fig. 4a,b). In contrast, vascular phenotyping revealed marked genotype-dependent effects. Systemically, infected *Rhbdl2*^{-/-} mice displayed increased plasma levels of soluble ICAM-1 (sICAM-1), sVCAM-1, and D-dimer, indicating enhanced endothelial activation and coagulation (Fig. 4c). Notably, elevated sVCAM-1 levels were also observed in uninfected *Rhbdl2*^{-/-} mice, indicating endothelial alterations independent of infection.

***Rhbdl2* controls VE-cadherin integrity in the brain**

Given the endothelial phenotype, we examined known RHBDL2 substrates involved in junctional stability¹³. While BCAM expression was unchanged (Supplementary Fig. 2), *Rhbdl2* deficiency resulted in

increased *Cdh5* (VE-cadherin) gene expression but reduced levels of full-length VE-cadherin protein in the brain (120 kDa, Fig. 4d–f). Plasma VE-cadherin levels were not affected (Supplementary Fig. 3).

In conclusion, these data identify a locus in *RHBDL2* as a genetic determinant of cerebral infarction in pneumococcal meningitis and implicate *RHBDL2* as a mediator of endothelial remodeling and activation, linking host genetic susceptibility to altered protease activity, dysregulated endothelial function and junctional integrity.

This pathway provides a mechanistic framework connecting infection-driven inflammation to cerebrovascular complications.

DISCUSSION

Cerebral infarction represents one of the most frequent and devastating complications of pneumococcal meningitis, yet the host determinants underlying susceptibility to this complication have remained poorly defined. In this study, we identify genetic variation at the *RHBDL2* locus as a major determinant of cerebral infarction risk during pneumococcal meningitis and provide converging human and experimental evidence implicating endothelial junctional remodeling as a central pathogenic mechanism. By integrating genome-wide association data with cerebrospinal fluid biomarker profiling and mechanistic studies in a murine model, we establish endothelial protease signaling as a previously unrecognized pathway linking host genetic susceptibility to ischemic brain injury during bacterial meningitis.

Using an unbiased genome-wide approach, we identified a common intronic variant in *RHBDL2* that conferred a near fourfold increased risk of cerebral infarction, independent of established clinical predictors of disease severity⁸. The magnitude of this effect is notable in the context of infectious disease genetics, where effect sizes are typically modest^{14,15}, and underscores the importance of host vascular biology in shaping neurological outcome during severe infection. Risk allele carriers displayed a distinct cerebrospinal fluid profile characterized by elevated PAI-2 and soluble VCAM-1 levels, indicating enhanced antifibrinolytic activity and endothelial activation within the central nervous system. Both markers have previously been associated with adverse outcome in pneumococcal meningitis^{16,17}, supporting their relevance to cerebrovascular pathology. The absence of broad alterations in other coagulation markers suggests that the *RHBDL2*-associated phenotype reflects targeted perturbation of endothelial pathways rather than generalized coagulopathy.

RHBDL2 encodes a rhomboid intramembrane serine protease known to regulate membrane-bound substrates involved in endothelial signaling and junctional integrity¹³. Among its established targets are thrombomodulin and VE-cadherin, proteins that play critical roles in balancing vascular stability, inflammation, and coagulation^{18–20}. Although *RHBDL2* protein was not detectable in CSF of patients with meningitis, reduced levels of EGF, a known *RHBDL2* cleavage product, in risk allele carriers support altered *RHBDL2* proteolytic activity *in vivo*.

To interrogate the functional relevance of *RHBDL2* during infection, we employed a well-established mouse model of pneumococcal meningitis¹². Genetic deletion of *Rhbdl2* resulted in reduced bacterial burden in the brain and spleen, attenuated neuroinflammation, and decreased parenchymal leukocyte infiltration. Notably, overt cerebral infarction was not observed in this model, reflecting recognized limitations of current experimental systems in recapitulating late-stage cerebrovascular complications seen in humans. Nevertheless, vascular phenotyping revealed marked genotype-dependent alterations, including systemic endothelial activation, increased D-dimer levels and reduced VE-cadherin abundance in brain tissue, supporting a role for *Rhbdl2* in regulating endothelial homeostasis during infection.

At first glance, the partially protective phenotype observed in *Rhbdl2*-deficient mice appears paradoxical in light of the increased infarction risk associated with the human *RHBDL2* variant. However, the intronic risk allele identified here is likely to exert regulatory and context-dependent effects rather than complete loss of function, potentially leading to partial or spatially restricted alterations in protease activity. In addition, genetic deletion may provoke compensatory engagement of alternative sheddases, such as ADAM10 or ADAM17,^{21,22} that remodel endothelial junctions through parallel pathways. These considerations highlight the complexity of protease-mediated vascular regulation and caution against simple extrapolation from knockout models to human regulatory variants.

Together, these findings support a model in which *RHBDL2* acts as a rheostat of endothelial remodeling during infection, balancing junctional stability, inflammatory activation and fibrinolytic activity. Genetic perturbation of this pathway appears to shift this balance toward a pro-thrombotic, activated endothelial state, thereby increasing vulnerability to ischemic injury in the inflamed cerebral vasculature. Such a framework integrates previous observations linking coagulation and endothelial dysfunction to adverse outcome in bacterial meningitis and extends them by identifying a specific protease-mediated regulatory node.

Our study has limitations. First, replicating of our genetic findings in independent cohorts was not feasible owing to the rarity of pneumococcal meningitis cohorts with detailed neuroimaging and genomic data; future international collaborative efforts will be essential to validate and extend these observations. Although our study represents the largest genetic analysis of pneumococcal meningitis to date, the modest number of infarction events limits detection of variants with smaller effects. Additionally, although our study is the largest cohort study on pneumococcal meningitis to date, the number of patients included in our association study is relatively low. However, the credibility of the association is reinforced by genome-wide significance, a large effect size, consistent clinical correlates, genotype-dependent biomarker changes in human cerebrospinal fluid, and mechanistic validation *in vivo*. Lastly, while the intronic variant identified here implicates *RHBDL2*, further studies in vascular tissues will be required to define the precise regulatory mechanisms linking genotype to protease activity.

In conclusion, we identify *RHBDL2* as a previously unrecognized host genetic determinant of cerebrovascular complications in pneumococcal meningitis and delineate endothelial protease signaling as a central pathway connecting host susceptibility to vascular injury in severe infection. These findings

advance understanding of meningitis-associated cerebral infarction and suggest that modulation of endothelial remodeling pathways may represent a therapeutic strategy to mitigate cerebrovascular complications in infectious and inflammatory diseases of the central nervous system.

METHODS

Nationwide clinical cohort

The MeninGene study is a nationwide prospective cohort study on community-acquired bacterial meningitis in the Netherlands¹. Methods of patient identification and inclusion have been published previously¹. In short, we included patients aged 16 years or older who had pneumococcal meningitis between March 2006 and July 2021. Patients were identified by the treating physician or the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM). Extensive clinical data was prospectively collected through online case record forms. We included all patients with confirmed pneumococcal meningitis, identified either by the presence of *S. pneumoniae* in the CSF or by a combination of a positive PCR, blood culture, or CSF antigen test along with at least one predictor of bacterial meningitis, as outlined by Spanos et al.²³. These predictors include a CSF glucose concentration < 340 mg/L (1.9 mmol/L), a CSF:blood glucose ratio < 0.23, protein concentration > 2200 mg/L, white blood cell count > 2000 per microliter, or > 1180 polymorphonuclear leukocytes per microliter. We excluded patients who developed bacterial meningitis in the hospital within one week of discharge, following head trauma or neurosurgery within the prior month, or those with a neurosurgical device in place. Patients with an altered immune status owing to asplenia, diabetes mellitus, cancer, alcoholism, or the use of immunosuppressive drugs were considered immunocompromised, as were patients infected with human immunodeficiency virus. Neurological examination was performed at discharge. Outcome was scored according to the Glasgow Outcome Scale (GOS), with scores varying from 1 (death) to 5 (mild or no disability), and then dichotomized in favorable (GOS 5) and unfavorable outcome (GOS 1-4)²⁴.

DNA extraction and genotyping

Methods on DNA extraction and genotyping for this study have been published elsewhere²⁵. Briefly, blood from patients was collected in sodium/EDTA tubes and sent to Amsterdam UMC, location AMC, for DNA isolation with the Gentra Puregene Isolation Kit (Qiagen). Genotyping was performed on the Illumina Omni array (Illumina). Genotypes were called from normalized intensity data using optiCall²⁶. Samples with a heterozygosity rate three standard deviations away from the mean or > 3% missing genotypes were removed. Markers with > 5% missing genotypes, minor allele frequency (MAF) < 1% or out of Hardy-Weinberg equilibrium (HWE; $p < 10^{-5}$) were removed. Sample relatedness was estimated with KING using an LD-pruned set of markers, and duplicate samples were removed²⁷. Intensity plots were manually inspected for associated markers using Evoker and miscalled sites were removed²⁸. Markers were reported with respect to the reference allele and coordinates of GRCh37. Markers were

imputed using the Haplotype Reference Consortium (HRC) panel as a reference with the Sanger Imputation Server²⁹⁻³¹. Again, markers with MAF < 1%, HWE $p < 10^{-5}$ or INFO scores < 0.7 were removed.

Genome-wide association analysis

The association study was performed in bolt-Imm (version 2.3.4), using the LD-pruned set of genotype markers to estimate the kinship matrix, and calculating association statistics for all genotyped and imputed sites passing quality control thresholds. We used age as a covariate in the analysis. Manhattan plots were generated with the qqman package in R, and locus plots with locuszoom (version 1.4, using the hg38 build European population as reference assembly).

CSF measurements

EGF, fibrinogen, PAI-1, PAI-2 and sVCAM-1 were measured in CSF from a subset of 192 of the 744 genotyped patients. Thrombomodulin was measured in an independent subset of 183 genotyped patients. All measurements were performed using enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (Merck Millipore for fibrinogen, PAI-1, PAI-2, sVCAM-1 and EGF; Abcam for thrombomodulin).

Bacterial strain and culture

All procedures related to pathogenic bacteria were conducted under biosafety level 2 protocols and guidelines. The clinical isolate *S. pneumoniae* D39 (serotype 2) from the Veening lab was used in this study. *S. pneumoniae* was grown in CY medium at 37 °C to mid-log phase, evaluated by monitoring at an optical density of 600 nm. Frozen stocks of bacteria in 20% glycerol (Sigma) were prepared and kept at -80 °C until use.

Mice

Wild-type mice were purchased from vendor (C57BL/6NCrl, C57BL/6NHsd or C57BL/6NRj) and *Rhbd12*^{-/-} mice (C57BL/6N-Atm1Brd Rhbd12tm1b(KOMP)Wtsi/JMmucd) mice were derived from cryo-preserved spermatozoa 049439-UCD-SPERM, provided by MMRRRC USA. Mice were housed at the Animal Research Institute Amsterdam UMC (ARIA). Knockout of *Rhbd12* in *Rhbd12*^{-/-} mice was validated by measuring gene expression in brain and kidney tissue by reverse transcription quantitative polymerase chain reaction (RT-qPCR; Supplementary Fig. 4). Animals (maximum of 6 mice/cage) were housed in individually ventilated cages (Tecniplast 1284L Eurostandard Type II L) with corncob bedding in a ventilated cabinet with a controlled temperature of 20–24 °C, relative humidity 45-65% and 12 hours light/dark (07:00-19:00) cycles. Mice were given food and water (acidified; pH 2,3-2,8) *ad libitum*. The study was conducted in accordance with the ARRIVE guidelines for reporting animal experiments.

Mouse meningitis model

A well-characterized mouse model of pneumococcal meningitis was used³². Mice were acclimatized for one week prior to experiments and housed in individually ventilated cages with corncob bedding under controlled conditions. Researchers were blinded for genotype during experiments and data analysis. On day 0 (start of the experiment) mice were weighed and clinically scored. Clinical scoring included weight loss (0–4 points), activity (0–3 points), time to return to upright position (0–6 points), state of skin/fur (0–2 points), posture (0–2 points), eye discharge or protrusion (0–4 points), irregular/labored breathing (0–4 points), presence of seizures, limb paresis or ataxia (0–6 points; Supplementary Table 3). Animals reaching humane endpoint (HEP) criteria (clinical scoring ≥ 14 , weight loss $\geq 25\%$, coma, paralysis, seizure longer than 5 min. or two seizures in 15 minutes) were terminated and excluded from data analysis. Subsequently, mice were inoculated with 1 μ l bacterial suspension containing 5.0×10^4 CFU *S. pneumoniae* (infection group) or PBS (uninfected controls) into the cisterna magna under isoflurane anesthesia. Shortly after intracisternal inoculation mice were assessed for neurologic damage as a result of the puncture using the Foot Fault Grid test. Mice suffering from neurological damage as a result of the puncture were excluded. Treatment with antibiotics was started 20 hours post inoculation (hpi) with bacteria by intraperitoneal injection of ceftriaxone (100 mg/kg body weight) and repeated after 12 hours. Clinical scoring was performed every 4 hours from 16 hpi onwards until the end of the experiment or until a HEP was reached. Mice reaching a HEP were scored as 15 points for the remainder of the study. At the experimental endpoint of the study mice were anesthetized by intraperitoneal injection of 190 mg/kg ketamine + 0,3 mg/kg dexmedetomidine in a total volume of 300 μ l, followed by cardiac puncture for blood collection and perfusion of organs with sterile PBS via the left ventricle. The right hemisphere of the brain was placed in formalin. At 20 hpi, the left hemisphere of the brain and the spleen were placed in ice-cold 0.9% saline solution, processed and stored as described previously¹², while at 44 hpi the left hemisphere of the brain was snap-frozen in liquid nitrogen and used for counting cerebral hemorrhages. Bacterial titers were determined by plating serial tenfold dilutions of blood, CSF, brain and spleen homogenates on sheep-blood agar plates and incubated overnight at 37 °C. EDTA blood was centrifuged at 2000 g for 15 minutes. Plasma was stored at -70°C for further analysis. Outcome measures included clinical scores, CFU counts, brain histology scores, cytokine concentrations and gene expression in brain, spleen, and plasma.

Experimental groups in the mouse model

The following experimental groups were used each time point: uninfected wild-type mice (N = 12, 6 males + 6 females), uninfected *Rhbdl2*^{-/-} mice (N = 12), infected wild-type mice (N = 24) and infected *Rhbdl2*^{-/-} mice (N = 24). Half of the mice were terminated at 20 hpi (N = 36), the other half at 44 hpi (N = 36). The study was powered to detect a difference in inflammatory parameters between groups with an effect size ($\delta = |\mu_1 - \mu_2|/\sigma$) of 1.3. Using an 80 % power, two-sided testing, and significance level of $p < 0.05$, we needed 12 mice per group. Experiments were divided over two sessions, using 36 mice with equal representation of experimental groups in both sessions.

Immunohistochemistry and histopathology

Histopathology was performed on the right hemisphere of the brain. Brain was fixed in 4% paraformaldehyde and paraffin-embedded in seven coronal plaques which were cut in sections of 5 µm. Samples were stained with haematoxylin-eosin (HE) with the Ventana BenchMark ULTRA system (Roche). Immunostaining was performed with antibodies against CD45 (Biolegend) to detect leukocytes followed by a haematoxylin counterstain. Histopathology was scored (blinded) for six categories by two researchers separately as previously described³³. Discrepancies in scoring were resolved through consultation with a dedicated neuropathologist. A detailed description of the histological scoring method can be found in Supplementary Table 4.

Analysis of cerebral bleeding

The left hemisphere of the brain of mice terminated at the 44 hour time point was dissected and cut into 50-µM-thick sections. Starting at the anterior part of the brain, 9 serial sections were photographed with a digital camera at an 0.3 mm interval. In each photograph the number of hemorrhagic spots were counted and the hemorrhagic area was measured according to the protocol of Tang *et al.*, (2010) using Image J (v1.50i)³⁴.

Blood-brain barrier permeability

Blood–brain barrier (BBB) permeability was assessed by immunohistochemical detection of endogenous IgG in murine brain sections. After deparaffinization and antigen retrieval, sections were incubated with an HRP-conjugated anti-mouse IgG secondary antibody (DPVB110HRP, Immunologic). Signal was visualized using DAB (VWRKBS04, VWR). Slides were imaged with a Mica Microhub microscope (Leica). Quantification of total tissue area and DAB positive area was performed in ImageJ by thresholding. The percentage of DAB positive area relative to total tissue area was calculated and used as a measure of blood-brain barrier permeability. Non-infected mice served as controls.

Isolation of bone marrow-derived neutrophils (BMDNs) and macrophages (BMDMs)

Bone marrow cells were isolated from femurs and tibias from uninfected wild-type and *Rhbd12^{-/-}* mice by flushing the bones with ice cold DPBS (-/-). Collected cells were filtered through a 70 µM mesh filter and counted, followed by diluting to a concentration of 25×10^7 cells/mL. Neutrophils were isolated by negative magnetic selection using the MACS separation system, according to the manufacturers' instructions (Miltenyi Biotec). In short, cells were incubated with Neutrophil Biotin-antibody Cocktail for 10 min at 4°C, washed and then incubated with anti-biotin Microbeads for 15 min at 4°C. Following incubation, cells were washed, centrifuged and resuspended, followed by magnetic separation on a MACS column. Unlabeled neutrophils were collected, washed and resuspended in RPMI without phenol red and 0.1% BSA to the desired concentration for downstream experiments. Neutrophil enrichment was confirmed by quantifying the number of CD11b⁺Ly6G⁺ cells using flow cytometry. Labelled cells were flushed from the column, washed and cultured at 37°C and 5% CO₂ for 8 days in Dulbecco's Modified Eagle Medium (DMEM; Low glucose, ThermoFisher) supplemented with 10% fetal calf serum (FCS), 1%

penicillin/streptomycin, 2 mM L-glutamine and 15% L929-conditioned medium (LCM) for differentiation into bone marrow-derived macrophages (BMDMs). Fresh medium was added on day 3 and day 7. On day 8, differentiated cells (>95% of cells CD11b⁺F4/80⁺ determined by flow cytometry) were harvested and seeded in DMEM supplemented with 10% FCS and 2 mM L-glutamine at a concentration of 0.75×10^6 cells/ml for extracellular flux analysis and 1.25×10^6 cells/ml for all other experiments. Cells were left to attach for 24 hours before performing experiments. BMDMs were stimulated for 4 or 24 hours with LPS (10 ng/ml), LPS (10 ng/ml) + IFN γ (10 ng/ml), *S. pneumoniae* D39V (MOI 1) or left unstimulated by adding fresh DMEM. For experiments lasting longer than 4 hours, fresh medium was added containing penicillin-streptomycin (final concentration 1%) after 4 hours.

Western blotting

For western blot analysis, total protein was extracted from brain tissue using RIPA buffer. Protein concentrations were determined with BCA Protein Assay (ThermoFisher). SDS-polyacrylamide gels (Bio-Rad) were used to separate 50 ug of protein, followed by transfer to PVDF membranes (ThermoFisher). Membranes were blocked in 5% goat serum (Biowest) for 1 hour at room temperature and subsequently incubated with primary antibodies against VE-cadherin (1:100, Invitrogen, 14-1441-82), β -tubulin (1:5000, ProteinTech, 10094-1-AP) and transferrin (1:4000, Invitrogen, PA5-27306). Overnight incubation was performed at 4°C. Next, membranes were washed and incubated with a HRP-conjugated secondary antibody (1:5000, ThermoFisher, G-21234 or 1:2500, Invitrogen, 31470), followed by visualization with an ECL detection system (ThermoFisher). Images were acquired using the Platinum V10 (Uvitech).

Nitric oxide assay

For the nitric oxide (NO) assay, 50 μ l of undiluted cell supernatant was transferred to a flat-bottom 96-wells plate. Next, 50 μ l of Griess reagent (Sigma) was added to the samples and absorbance was immediately measured on a microplate reader at a wavelength of 550 nm. To calculate exact concentrations of NO, a standard curve was made with NaNO₂ in culture medium.

Phagocytosis assay

BMDNs and BMDMs were incubated with pHrodo BioParticles (1 mg/mL, ThermoFisher) for 60 minutes at 37 °C, according to the manufacturer's instructions. As a negative control, cells were incubated in presence of 10 ug/mL cytochalasin D (ThermoFisher), an inhibitor of phagocytosis. Cells were analyzed by flow cytometry (BD Biosciences A1 Symphony).

ELISA measurements

In mice, cytokine concentrations of chemokine (C-X-C motif) ligand 2 (CXCL2), IL-1 β , IL-6, and TNF- α were determined in the supernatant or organ homogenates using ELISA, according to manufacturer's instructions (Duoset, R&D Systems).

Gene expression

Total RNA was extracted from the mouse brain and kidney using the Nucleospin RNA kit (Macherey-Nagel), according to the manufacturer's protocol. Quality of the samples and RNA concentrations were determined with a Nanodrop spectrophotometer (ThermoFisher). Following RNA extraction, cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The RT-PCR measurement of individual cDNAs was performed on a Bio-Rad MyiQ Single-Color RT-PCR Detection System using the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories). A list of primers that were used in this study can be found in Supplementary Table 5.

Migration assay

To assess migration of BMDNs, 5.0 μ M transwell inserts (Sarstedt) and 24-well plates were coated with 2% BSA (Merck) and incubated for 1 hour at 37°C to improve cell attachment. In the lower compartment, 800 μ L of medium containing 10 nM CXCL2 (Peprotech) was added as a chemoattractant and cells were seeded at a concentration of 200×10^3 cells per insert. To allow migration, plates were incubated for 4 hours at 37°C and 5% CO₂, after which both the number of cells in the upper and lower compartment were counted and used to calculate the percentage of migrated cells.

NETosis assay

The production of neutrophil extracellular traps (NETs) was determined upon infection with *S. pneumoniae*, using SYTOX Green dye (Invitrogen). In short, 1×10^6 BMDNs were incubated with 0.1 nM SYTOX green, followed by stimulation with 0.01% dimethyl sulfoxide (DMSO, Merck) as a negative control, 50 nM phorbol 12-myristate 13-acetate (PMA, Merck) as a positive control or *S. pneumoniae* (MOI 25). Technical quadruplicates were seeded at a concentration of 2×10^5 cells in a 96-well suspension plate and incubated for 2 hours at 37°C and 5% CO₂. The percentage of SYTOX green⁺ cells was determined using flow cytometry (BD Biosciences A1 Symphony).

Reactive oxygen species (ROS) assay

ROS production was assessed in BMDMs using CellROX Green Reagent (Invitrogen) according to the manufacturers' protocol. BMDMs were stimulated with either PBS as a negative control, *S. pneumoniae* (MOI 25) or 0.4 mM tert-butylhydroperoxide (TBHP, ThermoFisher), an oxidant, as a positive control. One condition contained TBHP stimulated cells pre-incubated with 2.5 mM N-acetyl cysteine (NAC, ThermoFisher) for 1 hour at 37°C and 5% CO₂ to prevent ROS production and served as a second negative control. Following 1 hour incubation with stimuli, CellROX was added at a concentration of 500 nM and cells were incubated for another 30 min at 37°C and 5% CO₂ in the dark. Cells were fixed in 4% PFA and the percentage of CellROX positive cells was determined using flow cytometry (BD Biosciences A1 Symphony).

Statistics

Patient data

Categorical variables were presented as counts with percentages, while continuous variables were expressed as medians with interquartile ranges (IQR). Comparisons of categorical variables were performed using Fisher's exact test, whereas differences in continuous variables were assessed using either the t-test or the Mann-Whitney U test when the data did not follow a normal distribution. Logistic regression was used to evaluate the association between predictors and unfavorable outcome. Odds ratios (ORs) were reported with 95% CIs. Statistical tests were two-tailed and p-values below 0.05 were considered statistically significant. In case of multiple testing, Benjamini & Hochberg correction was administered. Statistical analyses were conducted in R version 4.3.2 or Graphpad version 10.2.0.

Mouse data

In the murine meningitis model, groups were tested for normality with the Shapiro-Wilk normality test. Normally distributed groups were compared using a t-test, while not normally distributed data was compared using a Mann-Whitney U test. Histology scores between groups were compared with the Fisher's exact test.

Experiments using BMDMs were compared using a t-test. In all statistical tests, p-values below 0.05 were considered statistically significant. Statistical analyses were conducted in R version 4.3.2 or Graphpad version 10.2.0.

Declarations

Study approval

Patients or their legal representatives gave written informed consent for participation. The Medical Ethics Committee of the Amsterdam University Medical Center, location AMC, University of Amsterdam, the Netherlands, approved the human studies (approval number NL43784.018.13).

Animal experiments were authorized by the Centrale Commissie Dierproeven Netherlands (document no. AVD11400202013797) and the local Animal Welfare Body (AWB; document no. SP2400061 and SP2400540), and were conducted according to institutional guidelines

Data availability

Summary statistics for the results from the genome wide association study can be found at <https://doi.org/10.6084/m9.figshare.31974084>. GWAS array data and metadata can be requested at the European Genome-Phenome Archive (EGA) under accession number EGAS00001005993. All other data is available upon reasonable request.

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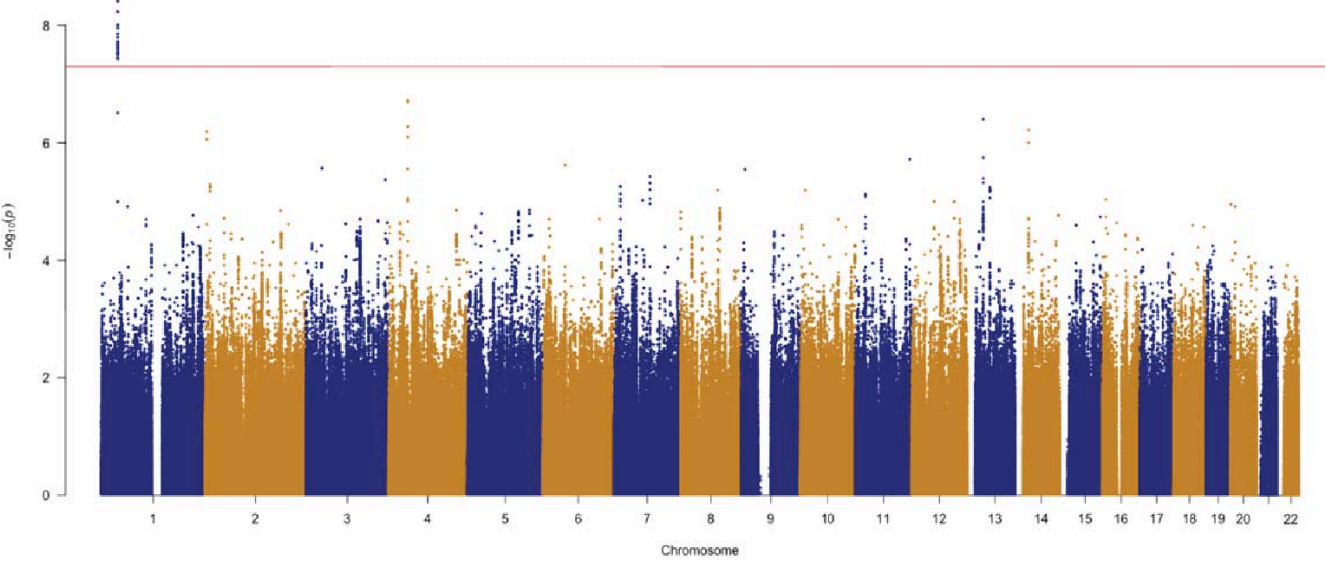
Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

Figure 1

a



b

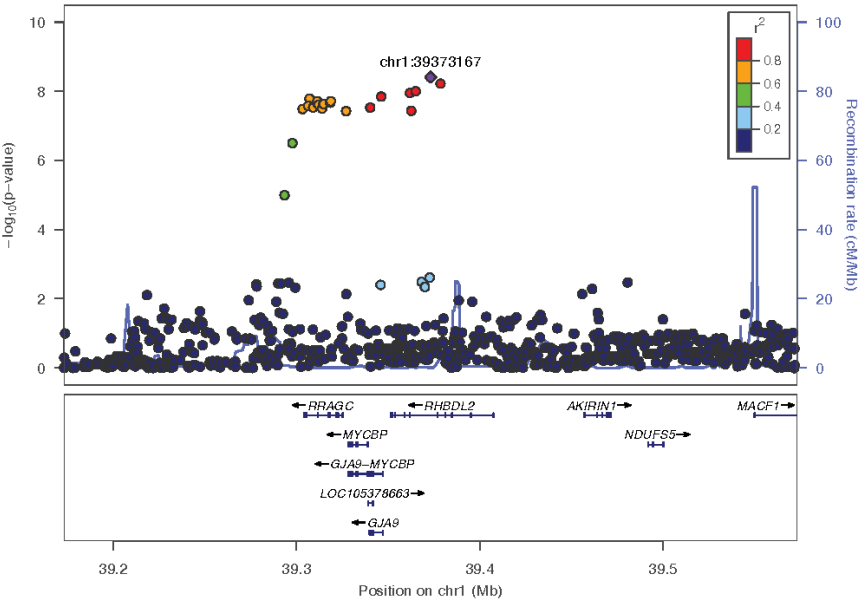


Figure 1

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Figure 2

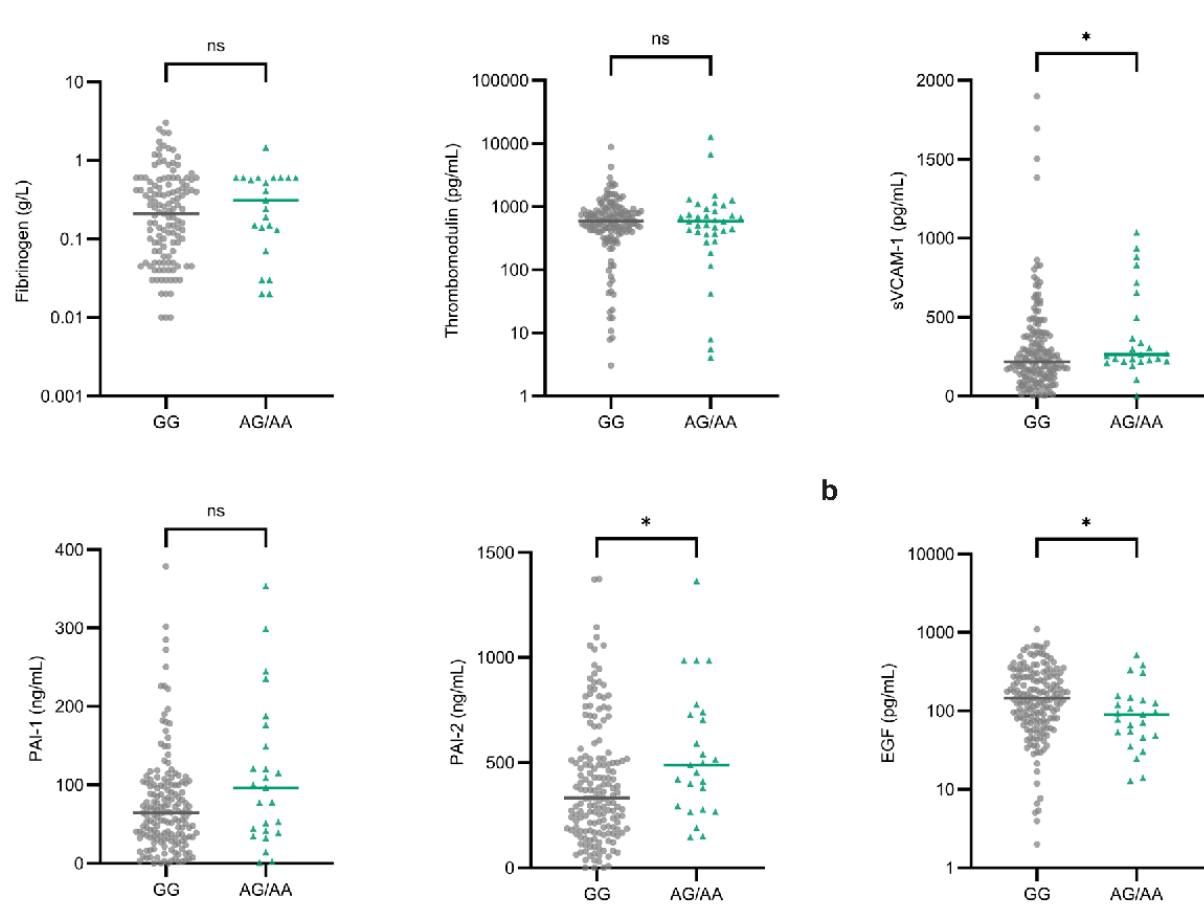
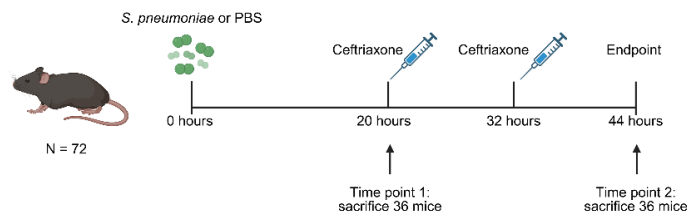


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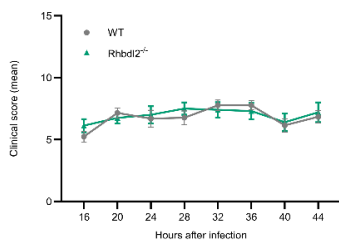
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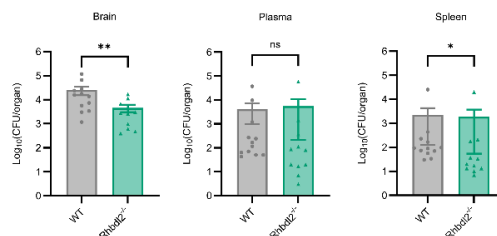
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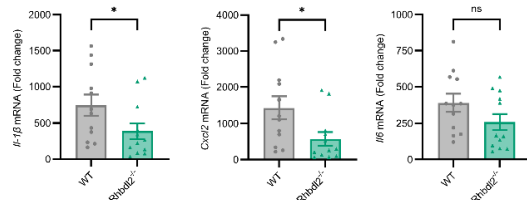
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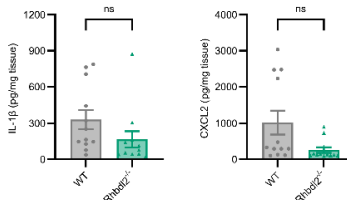
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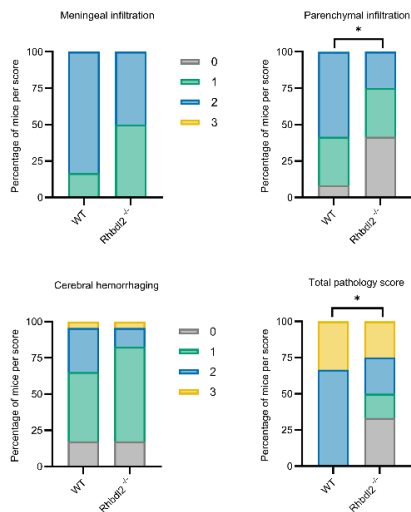
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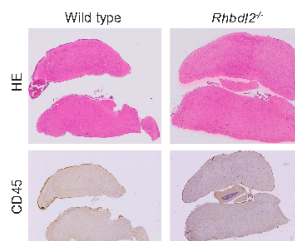
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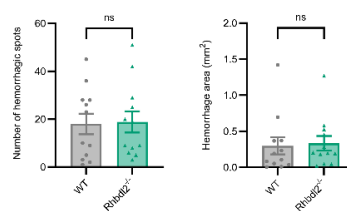


Figure 3

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Figure 4

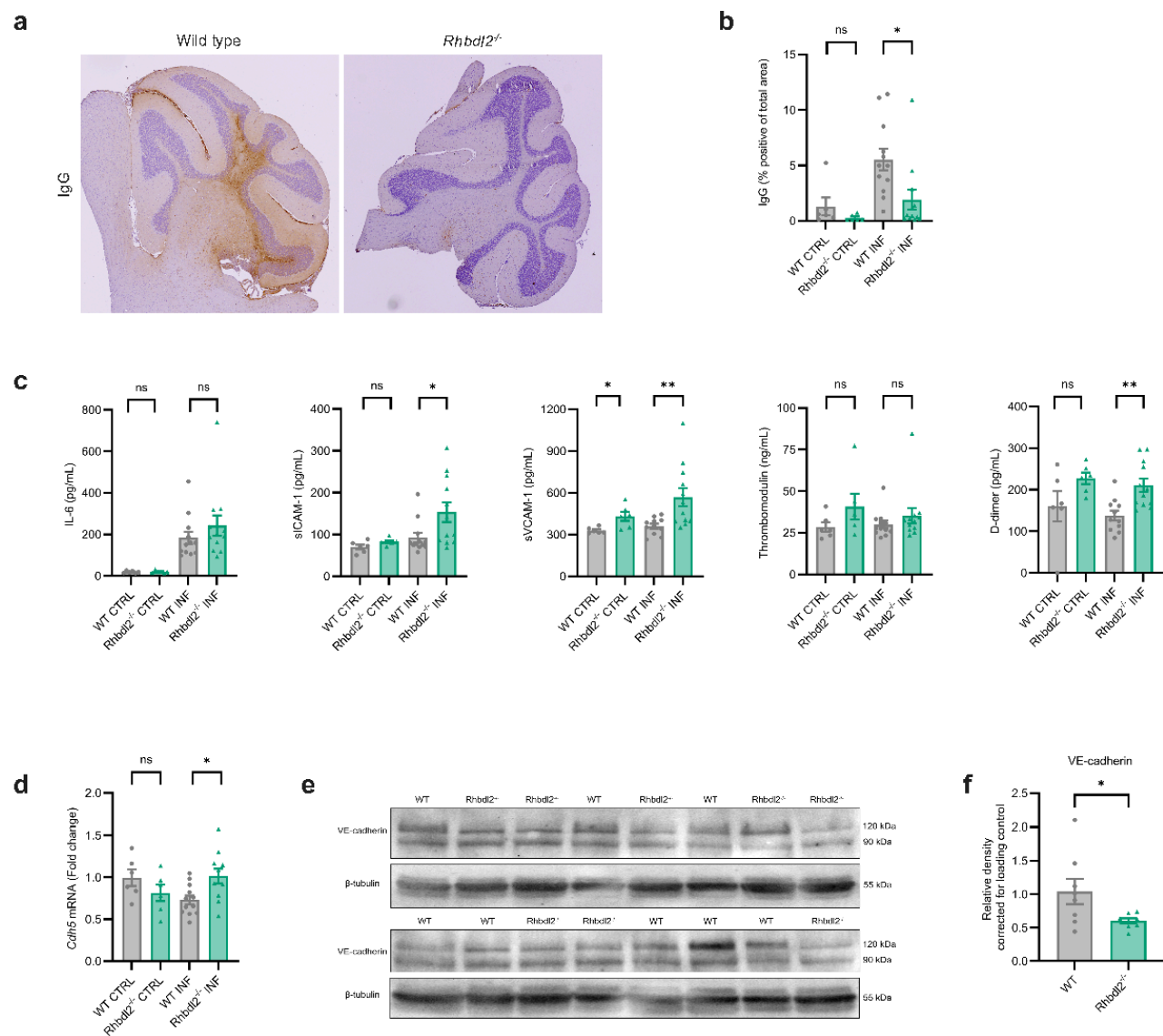


Figure 4

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