Vagal Signaling Decline in Age-related Macular Degeneration Drives Spleen-Dependent Retinal Inflammation

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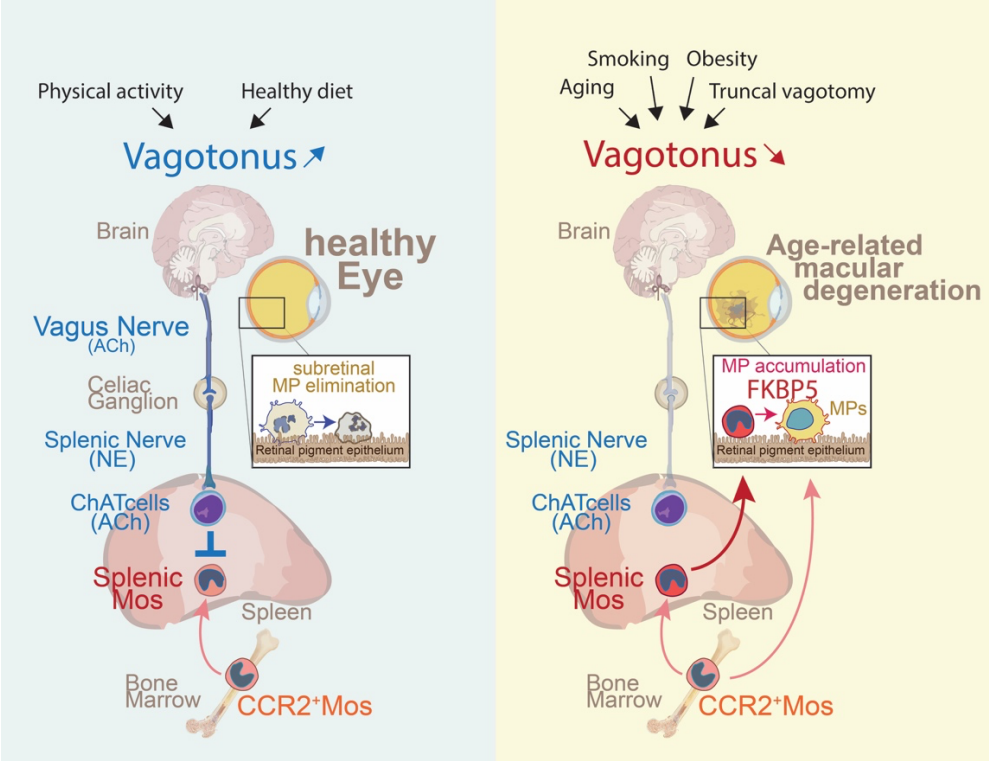
Abstract

Under relaxed physiological conditions, vagal innervation via the splenic nerve restrains the release of inflammatory cytokines from splenic macrophages and helps preserve systemic homeostasis, constituting the efferent cholinergic anti-inflammatory arm of the inflammatory reflex. Our analysis of Danish National Patient Registry data revealed that vagotomy for peptic ulcer disease, particularly truncal vagotomy, markedly increased the risk of developing age-related macular degeneration (AMD). Consistently, experimental truncal vagotomy or splenic denervation in mice exacerbated laser- and light-induced subretinal inflammation, both models of late AMD. The pro-inflammatory effects of vagotomy were abolished by concurrent splenectomy or pharmacologically augmenting acetylcholine signaling. Mechanistically, vagotomy activated a pro-inflammatory transcriptional program in splenic monocytes while suppressing tissue-retention genes *Cxcr4* and *Fn1*, leading to enhanced monocyte egress from the spleen and increased infiltration into the injured retina. In the laser-injured retina, single-cell RNA sequencing (scRNAseq) of mononuclear phagocytes revealed a broad set of vagotomy-induced transcripts in infiltrating monocytes and activated microglia. Remarkably, more than one-third of these were normalized by splenectomy. Together, these findings identify a vagus nerve–spleen–retina axis that connects stress and vagal tone to AMD pathogenesis.

Keywords (10 max)

age-related macular degeneration, neuroinflammation, monocytes, microglia, vagus nerve, vagal tone, inflammatory reflex, cohort study

Graphical abstract



In Brief

Using population-level human data and complementary mouse models, this study demonstrates that loss of vagal signaling enhances subretinal inflammation and increases susceptibility to age-related macular degeneration (AMD). Mechanistically, vagotomy reprograms splenic monocytes toward a pro-inflammatory state, promoting their egress and infiltration into the injured retina, where they amplify local inflammation. These effects identify a vagus nerve–spleen–retina axis that links reduced vagal tone to AMD pathogenesis.

Highlights

- Vagotomy increases AMD risk in humans and exacerbates subretinal inflammation in mouse models of late-stage AMD.
- Loss of vagal signaling drives pro-inflammatory reprogramming and enhanced egress of splenic monocytes via suppression of tissue-retention genes.
- Single-cell transcriptomics reveals vagotomy-induced inflammatory signatures in retinal monocytes and microglia that are largely reversed by splenectomy.

Background

Age-related macular degeneration (AMD) arises from age, genetic, and environmental factors. Early disease is marked by subretinal deposits (pseudodrusen and soft drusen), and progression leads to debilitating choroidal neovascularization (CNV, neoAMD) or an extending area of outer retinal degeneration (geographic atrophy, GA) (Guillonneau et al., 2017). VEGF inhibition suppresses CNV, but complement factor 3 or 5 blockade provides little benefit for neurodegeneration in GA, and all require burdensome lifelong repeated injections.

A shared pathogenic axis across AMD forms is chronic inflammation (Guillonneau et al., 2017; Yu et al., 2020). In AMD, microglia (MC) become activated and, along with monocyte-derived macrophages (MdMs), infiltrate the photoreceptor layer that is normally devoid of immune cells (Guillonneau et al., 2017; Lad et al., 2015; Sennlaub et al., 2013). The three major genetic risk factors, APOE2 (Levy et al., 2015), a CFH variant (Calippe et al., 2017), and 10q26-driven HTRA1 overexpression in MPs (Beguier et al., 2020), promote this MP accumulation (Beguier et al., 2020; Calippe et al., 2017; Levy et al., 2015). In early disease MPs may contribute to debris clearance, but in advanced AMD age-related, genetic, and environmental factors promote their pathogenic polarization. In animal models, infiltrating activated MPs release inflammatory cytokines and reactive oxygen species that drive CNV, accelerate photoreceptor loss, and amplify inflammation by recruiting additional MPs (Guillonneau et al., 2017). MdMs in general and particularly those derived from splenic monocytes, are especially pathogenic in these models (Roubeix et al., 2024; Sennlaub et al., 2013).

Evidence from humans supports similar mechanisms. Circulating monocytes from patients with AMD display elevated CCL2, IL-8, VEGFA, and IL-6 expression, among other transcriptional alterations (Grunin et al., 2016; Lechner et al., 2017). Serum IL-6 levels further correlate with AMD incidence and progression (Guillonneau et al., 2017; Nahavandipour et al., 2020) and aqueous humor IL-6 levels are increased in GA (Huang et al., 2025). Consistent with a pathogenic role of mononuclear phagocyte cytokines, Janus kinase (JAK) inhibitors, which block IL-6, GM-CSF, IFN-γ, and other pro-inflammatory cytokines signaling, are associated with reduced AMD incidence (Hallak et al., 2024). Collectively, these findings highlight both local and systemic inflammation as central drivers of AMD pathogenesis.

One important regulator of the immune system is the inflammatory reflex, a vagus nerve–mediated neuroimmune pathway that senses peripheral inflammation and suppresses excessive

95 cytokine production, thereby maintaining immune homeostasis. In its cholinergic anti-
96 inflammatory efferent arm, parasympathetic signals from the vagus nerve stimulate the splenic
97 nerve at the coeliac ganglion, increasing norepinephrine release in the spleen. This stimulates
98 acetylcholine release from choline acetyltransferase (ChAT)-expressing T cells, which activates
99 α7 nicotinic acetylcholine receptors on splenic macrophages, delivering a tonic inhibitory signal
100 that suppresses macrophage activity and limits the release of proinflammatory cytokines into the
101 circulation (Andersson & Tracey, 2012; Chavan et al., 2017). Disruption of this reflex by truncal
102 vagotomy in animals leads to disproportionate cytokine responses and increased mortality during
103 sterile endotoxic shock, whereas vagal nerve stimulation improves survival (Borovikova et al.,
104 2000). In humans, truncal vagotomy has been linked to an increased incidence of RA (Baker et al.,
105 2025), whereas implantable vagus nerve stimulation has been shown to reduce disease severity
106 and was recently granted FDA premarket approval (PMA number P240039) (Koopman et al.,
107 2016). It remains unclear, however, whether the protective effect of high vagal tone in RA is
108 mediated through regulation of systemic cytokine release or through other, as yet unidentified,
109 anti-inflammatory mechanisms.

110 Vagal tone diminishes with aging, smoking and obesity (Andersson & Tracey, 2012; Bodin
111 et al., 2017), the main non-genetic AMD risk factors (Fleckenstein et al., 2021), and increases with
112 aerobic exercise, which protects against AMD (Andersson & Tracey, 2012; McGuinness et al.,
113 2017). We recently demonstrated that reduced heart rate variability, reflecting impaired vagal tone,
114 is associated with neoAMD (Molbech et al., 2025). This observation suggests that individuals with
115 AMD may exhibit diminished vagal activity, which could contribute to a dysregulated
116 inflammatory reflex, heightened inflammatory responses, and progression of the disease.

117 Here we demonstrate that truncal vagotomy is associated with an increased risk of AMD
118 in humans, and that experimental vagotomy or splenic denervation in mice exacerbated subretinal
119 inflammation. These pro-inflammatory effects depended on an intact cholinergic anti-
120 inflammatory pathway and were abolished by splenectomy or by inhibition of acetylcholine
121 degradation. Mechanistically, vagotomy reprogrammed splenic monocytes and promoted their
122 mobilization, leading to exacerbated retinal inflammation, Together, these findings define a
123 vagus–spleen–retina axis that links vagal tone to splenic monocyte mobilization and pathogenic
124 macrophage infiltration in AMD pathogenesis, and highlight potential therapeutic targets.

Results

Vagotomy is associated with increased incidence of AMD

To investigate whether loss of vagal tone influences the risk of age-related macular degeneration (AMD), we performed a nationwide cohort study using Danish National Patient Registry data (Fig. 1A). Although advances in medical therapy, including antibiotics and proton pump inhibitors, have largely rendered vagotomy obsolete, the procedure was commonly performed for peptic ulcer disease in Denmark between 1977 and 1995 (Lagoo et al., 2014). Two vagotomy procedures were analyzed: truncal vagotomy (TV), which involves cutting the vagus nerves inferior to the esophageal hiatus and denervates major abdominal organs including the spleen, and superselective vagotomy (SSV), which cuts only gastric branches while largely sparing innervation of other abdominal organs, including the spleen (Fig. 1A). These procedures provided two patient groups with similar indications for surgery but differing degrees of systemic vagal denervation.

The study cohort included 9,695 patients who underwent vagotomy with at least 10 years of follow-up: 4,092 underwent TV, 5,245 underwent SSV, and 358 had unspecified vagotomy (Supplemental Table 1). A comparison cohort of 83,624 age- and sex-matched individuals from the general population was randomly sampled at a 10:1 ratio for each vagotomized patient. The index date for the comparison cohort was defined as the date of vagotomy in the matched patient. Patients with AMD diagnosed prior to the index date were not included in the study.

Over follow-up, vagotomy was associated with a higher cumulative incidence of AMD. After adjustment for age, sex, COPD (as a proxy for smoking), hypertension, obesity-related diseases, and 19 Charlson Comorbidity Index (CCI) categories, the overall adjusted hazard ratio (aHR) for AMD was 1.36 (Table 1, Figure 1B, Supplemental Table 2). When examining TV and SSV separately, SSV was associated with only a modest increase in AMD incidence (2.43 per 1,000 person-years vs. 2.01 in its comparison cohort), whereas TV was associated with a substantially higher incidence (3.82 per 1,000 person-years vs. 2.43 in its comparison cohort) and an aHR of 1.45 (Table 1, Figure 1C-D, Supplemental Table 2). Sensitivity analyses showed similarly elecated aHRs in the strata of patients without COPD (Supplemental Table 2).

The lack of a pronounced association in the SSV cohort suggests that the increased risk of AMD observed in TV patients is unlikely to be driven solely by peptic ulcer disease or by vagotomy-induced changes in gastric acidity, or other important peptic ulcer risk factors such as

156 smoking, which are common to both procedures. Instead, the data indicate that broader abdominal
157 denervation, including the splenic branch of the vagus nerve, may underlie the observed increase
158 in AMD incidence.

159 Vagotomy promotes inflammation and exacerbates disease severity in mouse models of
160 AMD in a spleen-dependent manner

161 Our data identified truncal vagotomy as an AMD risk factor, implicating reduced vagal
162 tone in disease pathogenesis, in agreement with evidence that the AMD-risk factors age, smoking,
163 and obesity depress vagotonus, whereas aerobic exercise protects against AMD and increases
164 vagotonus (Andersson & Tracey, 2012; Bodin et al., 2017; Fleckenstein et al., 2021; McGuinness
165 et al., 2017). Physiologically, parasympathetic vagal output activates the adrenergic splenic nerve
166 at the celiac ganglion, triggering acetylcholine release from ChAT^{+} T cells. This provides a tonic
167 inhibitory signal that suppresses proinflammatory cytokine production by splenic macrophages
168 and may exert additional spleen-dependent anti-inflammatory effects (Andersson & Tracey, 2012;
169 Chavan et al., 2017) (Fig. 2A). To model reduced vagal tone, we used left cervical truncal
170 vagotomy, which strongly reduces splenic nerve activity in mice (Carnevale et al., 2016).

171 In humanized transgenic mice expressing the AMD-risk APOE2 isoform (TRE2 mice),
172 vagotomy at 3 months of age increased age-associated subretinal IBA1^{+} MP accumulation that
173 characterizes this model (Calippe et al., 2017; Levy et al., 2015) measured at 13 months on
174 RPE/choroidal flatmounts (Fig. 2B). In the laser-induced subretinal inflammation and choroidal
175 neovascularization model (the standard model of neoAMD) in 3–month-old male C57BL/6J mice,
176 vagotomy (performed one month before laser to allow for complete recovery) increased IBA1^{+}
177 MP accumulation and CD102^{+} CNV area quantified on RPE/choroidal flatmounts. Concomitant
178 splenectomy normalized both parameters to levels observed in sham-operated controls,
179 demonstrating a spleen-dependent effect (Fig. 2C).

180 We next performed a series of experiments to determine if the pro-inflammatory effect of
181 vagotomy was mediated via the inflammatory reflex. First, denervating the spleen by ethanol
182 application at the splenic hiatus in 2-month-old male C57BL/6J mice (one month before laser to
183 allow for recovery), confirmed by loss of tyrosine-hydroxylase (TH) staining on spleen sections
184 (Fig. 2D, left), increased laser-induced IBA1^{+} MP accumulation and CD102^{+} CNV on
185 RPE/choroidal flatmounts relative to sham, phenocopying vagotomy and confirming splenic nerve
186 involvement (Fig. 2E, right). Next, vagotomy failed to augment subretinal MPs or CNV after laser

187 in 3-month-old male *Rag2*$^{-/-}$ mice, which lack mature lymphocytes including ChAT^{+} T cells but
188 continue to harbor pathogenic splenic monocytes (Roubeix et al., 2024) (Fig. 2E).
189 Next, mice received daily intraperitoneal injections of the acetylcholinesterase inhibitor
190 galantamine after laser to restore acetylcholine availability diminished by vagotomy and reduced
191 ChAT-cell output. This reversed the pro-inflammatory phenotype, returning subretinal MP counts
192 and CNV size to sham in the laser CNV model, similar to splenectomy (Fig. 2F). Further, 3-month-
193 old male TRE2 mice were exposed to a calibrated bright-light challenge, which induces MP
194 infiltration in TRE2 mice but not in controls and within a shorter time frame than in the
195 experiments shown in Fig. 2B (Calippe et al., 2017; Levy et al., 2015). Quantification of IBA1+
196 MPs on RPE flatmounts revealed that vagotomy also increased MP accumulation in this model,
197 an effect again abrogated by splenectomy and reversed by galantamine (Fig. 2G).
198 Taken together, our results demonstrate that disrupting vagus-to-spleen cholinergic
199 signaling through truncal vagotomy amplifies subretinal MP responses and CNV. This effect
200 requires both splenic innervation and lymphocytes, most likely ChAT^{+} T cells, and it can be
201 reversed by enhancing acetylcholine tone, consistent with the cholinergic efferent arm of the
202 inflammatory reflex. Notably, these findings in animal models parallel the clinical observations
203 that truncal vagotomy promoted AMD pathogenesis (Fig.1).
204 Loss of vagal tone reshapes the splenic monocyte transcriptome and drives their
205 mobilization
206 The spleen functions as a major reservoir of CCR2^{+}Ly6C^{high} monocytes (SpleMos)
207 (Swirski et al., 2009), which, together with circulating Ly6C^{high} monocytes, are recruited into
208 diseased tissues (Robbins et al., 2012; Swirski et al., 2009) where they differentiate into
209 inflammatory MdMs. We recently demonstrated that SpleMos play a particularly pathogenic role
210 in retinal inflammation in AMD (Roubeix et al., 2024), analogous to their established role in
211 atherosclerosis (Robbins et al., 2012). On this basis, we next examined cellular and molecular
212 changes in the spleen, focusing specifically on potentially pathogenic splenic monocytes following
213 the loss of vagal tone.
214 Flow cytometric analysis of spleens from 3-month-old mice, performed 30 days after
215 vagotomy, revealed no differences in the numbers of CD45^{+}CD11b^{+} MPs, CD45^{+}CD11b^{+}Ly6C^{high}
216 monocytes (Mos), or CD4^{+} and CD8^{+} T cells compared with sham-operated controls (Fig. 3A,
217 Supplemental Fig. 1). Bulk RNA sequencing of FACS-sorted CD45^{+}CD11b^{+}Ly6C^{high}Ly6G^{neg}

218 splenic Mos identified 508 upregulated and 1860 downregulated genes in splenic Mos from
219 vagotomized mice compared with sham-operated controls (Fig. 3B, Supplemental Table 5).
220 Classical monocyte markers, including *Ccr2*, *Ly6c2*, *Cd14*, and *Sell*, were expressed at comparable
221 levels between groups (Fig. 3C). In contrast, several genes associated with immune defense were
222 upregulated in Mos from vagotomized mice, such as MHC class II-related genes (*H2-Aa*, *CD74*)
223 and pattern recognition receptors (*Clec4g*, *CD209e*) (Fig. 3D). Several pro-inflammatory genes
224 were also upregulated, including *Il1b*, *Lilra5*, *Ccl4*, and *C5ar1* (Fig. 3E). Conversely, among the
225 1860 downregulated genes were several implicated in monocyte retention. These included two of
226 the most strongly regulated genes, *Fn1*, whose autocrine expression anchors endothelial cells
227 within the extracellular matrix (Cseh et al., 2010), and *Cxcr4*, a central mediator of bone marrow
228 monocyte retention (Chong et al., 2016), along with the integrins *Itga4* and *Itga1* (Fig. 3F).
229 To assess how vagal denervation affects monocyte efflux from the spleen, we analyzed
230 spleens of vagotomized mice with or without laser injury at day 1 (Fig. 3G). In the spleen, prior to
231 laser injury, the number of Ly6C^{high} monocytes did not differ between groups, as quantified by
232 flow cytometry. However, one day after laser treatment, spleens from vagotomized mice showed
233 a marked reduction in monocyte numbers, indicating enhanced efflux in response to injury (Fig.
234 3G).
235 Together, these findings suggest that disruption of vagal signaling alters the splenic
236 monocyte transcriptome toward enhanced immune defense and a pro-inflammatory profile,
237 potentially influencing their polarization upon infiltration into diseased tissues. Importantly,
238 vagotomy increased the mobilization of splenic Mos before the vagotomy-induced, spleen-
239 dependent increase in subretinal inflammation that we observed (Fig. 2).
240 Vagotomy induces transcriptional reprogramming of infiltrating monocytes and activated
241 microglia in the retina in a spleen-dependent manner
242 Since vagotomy altered the splenic monocyte transcriptome, enhanced their mobilization
243 (Fig. 3), and promoted spleen-dependent accumulation of chorio-retinal MPs (Fig. 2), we next
244 investigated how vagotomy-primed splenic monocytes influence the composition and polarization
245 of resident and infiltrating MPs during sterile chorio-retinal inflammation. We performed single-
246 cell RNA sequencing on sorted CD45^{+}CD11b^{+} cells from posterior eye cups from vagotomized or
247 sham operated mice one day after laser injury or without laser-injury (Fig. 4A). Using Seurat for
248 dimensionality reduction with principal component analysis (PCA), UMAP, and graph-based

249 clustering, we identified 11 transcriptionally distinct clusters of MPs based on canonical marker
250 gene expression (Fig. 4B). Under homeostatic non-laser conditions (Fig. 4C, upper panel), these
251 clusters were annotated as resident macrophages (*C1qa*^{high}, *Cd68*^{high}) of the choroid (*F13a1*^{high}) or
252 as microglial cells (MCs, *P2ry12*^{high}), as well as circulating monocytes (*Nr4a1*$^{+}$, *Spn*$^{+}$, *Ace*$^{+}$) that
253 segregated into two subsets: patrolling Mos (*Ly6c2*^{low}, *Ccr2*^{low}) and inflammatory Mos (*Ly6c2*^{high},
254 *Ccr2*^{high}). In post-lasered eyes, we additionally observed several subsets of activated MCs,
255 characterized by reduced expression of homeostatic MC genes and upregulation of inflammatory
256 cytokines (*Ccl12*, *Ccl2*) (clusters 2-5, Fig. 4C lower panel). We also detected a large population
257 of infiltrating Mos (cluster 9, *Lgals3*^{high}, *Il1b*^{high}, *Spn*^{low}, *Ace*^{low}), as well as early monocyte-derived
258 macrophages distinguished by increased *Itgax* (Cd11c, cluster 10 and 11) expression. It is
259 important to note that the Seurat-defined clusters are based on a broad transcriptional profile, and
260 these genes are shown here only as representative examples.

261 In non-lasered eyes, vagotomy did not alter the overall cell type composition compared to
262 sham-operated controls. The majority of MPs were homeostatic microglia, accompanied by a small
263 cluster of choroidal macrophages and predominantly patrolling monocytes (Fig. 4D). The
264 activation state of homeostatic microglia also remained unchanged, as evidenced by stable
265 expression of homeostatic markers, absence of activation marker expression, and only limited
266 transcriptional changes, with 20 genes upregulated and 24 genes downregulated between
267 vagotomy and sham conditions (Fig. 4E, Supplemental Table 6).

268 In sharp contrast, the lasered samples were dominated by activated microglia (clusters 2-
269 5) and infiltrating monocytes (clusters 9 and 10; Fig. 4C–D). Within the activated microglial
270 population, several subclusters were detected, but cluster #3 (activated MC 2) was markedly
271 expanded in the vagotomized condition compared to both sham and splenectomy controls,
272 prompting us to focus on this subset (Fig. 4F and G). Relative to sham/sham-operated laser-injured
273 animals, vagotomized/sham laser-injured mice displayed 162 upregulated and 95 downregulated
274 genes in the MC cluster 3 (Fig. 4F). Vagotomized/sham laser-injured mice displayed 74
275 upregulated and 24 downregulated genes relative to VGX/SpleX -operated laser-injured animals
276 (Supplemental Table 7). Strikingly, the vagotomy-induced deregulation of a large proportion of
277 genes (65/162 and 15/95, respectively) was reversed by splenectomy (Fig. 4F, Supplemental Table
278 8). The most regulated genes included *Il6ra*, *B2m*, *Srgn*, *Tlr7*, *C1qb*, and *H2-D1*, covering
279 pathways in cytokine signaling, antigen presentation, proteoglycan-mediated granule biology,

280 innate immune sensing, complement activation, transcriptional regulation, and MHC class II
281 presentation. Similarly, in infiltrating monocytes, we identified 330 upregulated and 82
282 downregulated genes in vagotomized/sham laser-injured mice, of which 105 and 21, respectively,
283 were normalized in VGX/SpleX laser-injured animals (Fig. 4G, Supplemental Tables 9-10). The
284 most regulated genes included *Jak2*, *Id2*, *Srgn*, *Vcan*, *Clec4d*, and *Filip1l*, which are associated
285 with cytokine signaling, transcriptional control, granule biology, extracellular matrix organization,
286 pathogen recognition, cytoskeletal regulation, and immune modulation.
287 Taken together, vagotomy had minimal impact on the transcriptome of MPs in uninjured
288 eyes. By contrast, following laser injury, which recruits, in part, spleen-derived monocytes
289 (Roubeix et al., 2024), vagotomy induced transcriptional changes in infiltrating monocytes but
290 also activated microglia. Notably, splenectomy reversed a substantial fraction of these
291 transcriptional changes.
292

Discussion

Factors known to reduce vagal tone, such as aging, smoking, and obesity (Andersson & Tracey, 2012; Bodin et al., 2017), are also established risk factors for AMD (Fleckenstein et al., 2021). In line with this, we recently reported that reduced heart rate variability, a marker of vagal tone, is associated with AMD (Molbech et al., 2025). In the present study, we demonstrated that the surgical vagotomy, once used in the treatment of peptic ulcers, increased the risk of AMD (all forms combined), independently of age, smoking, and obesity (Fig.1). Superselective vagotomy was associated with only a modest risk increase compared to the substantial risk increase of truncal vagotomy, supporting the interpretation that the observed association with truncal vagotomy is not attributable to peptic ulcer disease or related risk factors such as smoking, which are similarly distributed between the two patient groups.

Since truncal, but not superselective, vagotomy profoundly reduces vagal tone in the spleen, and truncal vagotomy (but not superselective) has recently been linked to the incidence of autoimmune rheumatoid arthritis (Baker et al., 2025), we hypothesized that the observed association of truncal vagotomy with AMD risk is driven by disruption of the anti-inflammatory effect of the vagus nerve on the spleen. Indeed, experimental cervical truncal vagotomy in mice, which markedly reduces splenic nerve activity (Carnevale et al., 2016), led to increased subretinal inflammation following laser injury, as well as exaggerated age-related MP accumulation observed in TRE2 mice expressing the AMD risk gene *APOE2* (Levy et al., 2015) (Fig. 2B-C). Importantly, splenectomy reversed the vagotomy-induced exaggerated inflammation in laser-induced subretinal inflammation and in light induced inflammation in *APOE2*-expressing mice (Fig. 2F-G), highlighting the spleen's pivotal role in mediating the proinflammatory effects of vagotomy.

We performed a series of experiments to examine if the pro-inflammatory effect of vagotomy is propagated via the disturbance of the inflammatory reflex (Fig. 2A). Splenic denervation alone was sufficient to amplify the laser-induced inflammatory response (Fig. 2D), suggesting that in our model parasympathetic anti-inflammatory influence outweighs the sympathetic tone transmitted through the splenic nerve, consistent with observations of splenic denervation in the endotoxin shock model (Rosas-Ballina et al., 2008). Acetylcholinesterase inhibitor galantamine administration, to restore acetylcholine levels decreased by both diminished vagotonus in the celiac ganglion and from $ChAT^+$ T cells in the spleen, also reversed the effect of vagotomy in both models (Fig 2F-G). Interestingly, use of anticholinergic drugs has been

324 previously associated with AMD (Aldebert et al., 2018), which our findings suggests might be
325 mediated at least in part by a loss of cholinergic signaling in the anti-inflammatory reflex.
326 The finding that vagotomy failed to augment laser-induced subretinal MPs or CNV in
327 *Rag2*$^{-/-}$ mice, which lack mature lymphocytes (Shinkai et al., 1992) including ChAT^{+} T cells but
328 still maintain splenic monocytes (Roubeix et al., 2024), further supports ChAT^{+} T-cell dependence
329 (Fig. 2E). However, in contrast to severe sterile endotoxic shock (Borovikova et al., 2000), we did
330 not observe vagotomy-induced excessive cytokine release by splenic macrophages (data not
331 shown) likely because localized ocular laser injury was not sufficient to increase cytokine release
332 from splenic macrophages contrary to endotoxic shock. Taken together this set of experiments
333 demonstrates that experimental vagotomy in mice aggravates AMD models and confirms the
334 clinical observation of truncal vagotomy association with AMD. Further, it establishes a pivotal
335 role of the spleen in mediating this effect.
336 In the spleen, under homeostatic conditions and in the absence of laser injury, vagotomy
337 did not alter the numbers of MPs, T cells, or monocytes (Fig. 3A, Supplemental Fig. 1). Given that
338 CD45^{+}CD11b^{+}Ly6C^{high} splenic monocytes play a particularly pathogenic role in retinal
339 inflammation in AMD (Roubeix et al., 2024), we next analyzed their transcriptome. Several pro-
340 inflammatory genes (*Il1b*, *Lilra5*, *Ccl4*, and *C5ar1*), as well as genes linked to immune defense
341 (MHC class II genes and pattern recognition receptors), were upregulated in splenic monocytes
342 after vagotomy compared to sham-operated animals (Fig. 3B-E). These findings raise the
343 possibility that vagal innervation provides a tonic inhibitory input to splenic monocytes, potentially
344 mediated by currently unidentified signals from splenic macrophages, beyond its previously
345 established effects on macrophage cytokine release (Andersson & Tracey, 2012; Chavan et al.,
346 2017). Notably, the most strongly regulated genes included downregulation of *Cxcr4*, a central
347 mediator of bone marrow monocyte retention (Chong et al., 2016) and Fibronectin 1 (*Fn1*), whose
348 autocrine expression anchors endothelial cells within the extracellular matrix (Cseh et al., 2010),
349 along with fibronectin-binding *Itga4* and collagen-binding *Itga1* (Fig. 3F). Given that the spleen
350 serves as a major reservoir for Ly6C^{high} monocytes (SpleMos) (Swirski et al., 2009), we next tested
351 whether these transcriptional changes translated into altered monocyte egress during
352 inflammation. Flow cytometric analysis revealed a marked reduction in splenic Ly6C^{high}
353 monocytes in vagotomized mice compared with sham-operated controls one day after laser injury,
354 consistent with enhanced egress (Fig. 3G).

355 Because vagotomy reprograms splenic monocytes, increases their mobilization (Fig. 3),
356 and amplifies spleen-dependent subretinal MP accumulation (Fig. 2), we next examined how these
357 primed cells influence MP composition and polarization during sterile chorio-retinal
358 inflammation. In eyes without laser injury, vagotomy produced only minimal transcriptional
359 alterations in the homeostatic microglia and choroidal macrophages (Fig. 4B-E). In contrast, laser-
360 induced injury led to the infiltration of monocytes and the appearance of several activated
361 microglial subtypes (Fig. 4B-D). Notably, vagotomy selectively increased the relative abundance
362 of activated MC2 (cluster #3) in a spleen-dependent manner, demonstrating that signals derived
363 from the spleen are required for this effect. A plausible possibility is that infiltrating spleMos
364 convey these signals; however, directly and specifically labeling spleMos remains technically
365 challenging. Subcapsular red pulp dye injections, similarly to intravenous injections, also label
366 classical circulating blood monocytes. Likewise, spleMos from transplanted GFP^{+} spleens are
367 replaced by GFP^{-} recipient spleMos before the host has fully recovered from surgery. Finally,
368 photoconversion of subcapsular spleMos in *Cx3cr1*^{Dendra2} mice (Miller et al., 2021) proved either
369 inefficient or excessively damaging at the ultraviolet intensities required in our hands. Technical
370 advancements and further studies will be necessary to decipher the specific molecular and
371 signaling pathways by which spleMos or other signals from the spleen modulate microglial
372 activation after vagotomy.
373 In both infiltrating monocytes and activated MCs, the differentially expressed genes
374 regulated by vagotomy were predominantly upregulated. Strikingly one-third of these changes
375 reverted to baseline after splenectomy, underscoring the necessity of the efferent arm of the
376 inflammatory reflex and the spleen.
377 Together, our demonstration of the involvement of the inflammatory reflex in AMD
378 pathogenesis points to several novel therapeutic strategies. In addition to pharmacological
379 inhibition of acetylcholinesterase the avoidance of anticholinergic drugs, our findings suggest that
380 AMD patients may benefit from therapeutic vagus nerve stimulation, which has been shown to
381 reduce neuroinflammation and modulate microglial polarization in rodent models of spinal cord
382 injury (Chen et al., 2022) and stroke (Zhang et al., 2021).

383 ## Conclusions

384 In conclusion, we demonstrate that vagotomy is associated with an increased
385 incidence of AMD in a human patient cohort and exacerbates pathogenic inflammation in mouse

386 models of the disease. Mechanistically, this effect stems from an exaggerated, spleen-dependent
387 inflammatory response that profoundly alters the chorioretinal mononuclear phagocyte response
388 during retinal inflammation. The pro-inflammatory consequences of experimental vagotomy can
389 be mitigated by splenectomy and pharmacological enhancement of parasympathetic signaling.
390 Together, these findings provide mechanistic insight into how systemic factors such as aging and
391 obesity may increase the risk of AMD, and they confirm cholinergic drugs and vagus nerve
392 stimulation as potential therapeutic strategies to dampen excessive inflammation in AMD and
393 other conditions associated with dysregulation of the inflammatory reflex.

394 ## Methods

395 ### Danish National Patient Registry cohort study

396 We conducted a population-based nationwide cohort study using Danish administrative
397 registry data, following principles similar to those in previous epidemiological studies (Svensson
398 et al., 2015). Denmark's tax-funded healthcare system provides all residents with free, unlimited
399 access to diagnostic work-up and treatment, including for AMD. The Civil Registration System
400 (CRS) assigns all residents a unique identification number, enabling linkage across national
401 registries (Schmidt et al., 2014; Schmidt et al., 2019).

402 All patients undergoing vagotomy (TV or SSV) between January 1, 1977 and December
403 31, 1995 were identified via procedural codes in the Danish National Patient Registry (DNPR;
404 Supplemental Table 4). AMD cases were identified with ICD-8 and ICD-10 codes (Supplemental
405 Table 3). Although surgical procedures are generally coded with high accurary, no validation
406 studies have specifically assessed the accuracy of DNPR procedure codes for vagotomy, so
407 although unlikely misclassification is therefore possible. However, the most likely
408 misclassification, if any, would be the coding of a vagotomy despite incomplete resection. Thus,
409 misclassification, if any, is expected to bias estimated associations towards the null (Svensson et
410 al., 2015).

411 For each vagotomized patient, ten age- and sex-matched individuals from the general
412 population who had not undergone vagotomy were randomly sampled with replacement. The index
413 date for each control was defined as the date of vagotomy in the matched patient. Patients in the
414 comparison cohort who underwent vagotomy after the index date were reclassified into the
415 vagotomy cohort. Individuals with pre-existing AMD were not included in the cohorts.

416 Comorbidities were assessed via linkage to Danish National Patient Registry data
417 according to 19 major disease categories from the Charlson Comorbidity Index (CCI;
418 Supplemental Table 3). Peptic ulcer disease was excluded from the CCI because it was present in
419 nearly all vagotomized patients. Comorbidity burden was classified as normal (0), moderate (1–
420 2), or severe (3+), according to the number of comorbid categories present.

421 All participants were followed from the index date until AMD diagnosis, death, emigration,
422 or December 31, 2012, whichever occurred first. Cumulative incidence curves were generated
423 using Kaplan-Meier analysis with death treated as a competing risk. Because proportional hazards
424 assumptions were not met in the first 10 years, analyses were restricted to patients surviving ≥10

425 years after vagotomy and their matched controls. Hazard ratios for AMD were estimated with Cox
426 proportional hazards regression, adjusted for age, sex, COPD, hypertension, obesity-related
427 diseases, and CCI category. Sensitivity analyses were performed, stratified by COPD as a proxy
428 for smoking, by obesity-related diseases, and by hypertension. E-values were calculated to assess
429 the potential effects of unmeasured confounders, such as smoking(VanderWeele & Ding, 2017).
430 All analyses were carried out in SAS v9.4 (Carey, NC). The study was approved by the
431 Danish Data Protection Agency (2015-57-0002 and 2016-051-000001).

432 Animals

433 C57BL/6J mice (Jax strain #000664) were purchased from the Janvier Breeding Center.
434 *TRE2* mice, which express the human APOE2 isoform, were engineered using targeted
435 replacement as previously described (Levy et al., 2015). *Rag2*$^{-/-}$ mice were purchased from Charles
436 River Laboratories (Jax strain #008449). All mice were negative for the *Crb1*^{rd8}, *Pde6b*^{rd1}, and
437 *Gnat2*^{cpfl3} mutations. Mice were housed under specific pathogen-free conditions in a 12 hr/12 hr
438 light/dark cycle with water and food available ad libitum. All experimental protocols and
439 procedures were approved by the French Ministry of Higher Education, Research and Innovation
440 (APAFIS authorization #19412 2019022215422220).

441 Surgeries (vagotomy, splenectomy, splenic denervation, and sham)

442 Mice received a subcutaneous injection of buprenorphine (0.05 mg/kg) 30 minutes before
443 surgery, then were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg).
444 Mice received a second subcutaneous injection of buprenorphine (0.05 mg/kg) the day after
445 surgery. Antiseptic solution (Vetedine, povidone iodine) was applied to all skin sutures
446 immediately after closure. Mice were allowed to fully recover for at least 1 month following
447 surgery before any further experiments.
448 For vagotomies, a 1 cm midline cervical incision was made, the left vagus nerve was cut,
449 and then the incision was closed with absorbable sutures. For sham vagotomies the left vagus nerve
450 was visualized but not cut, and were otherwise identical.
451 Splenectomies and sham splenectomies were performed as previously described (Roubeix
452 et al., 2024). In brief, a 1 cm incision was made on the left side of the abdomen under the rib cage,
453 the spleen was removed by cutting the connective tissue and cauterizing the splenic vessels (or
454 only visualized without removal for sham splenectomies), and finally the incisions in the
455 abdominal muscles and skin were each closed with absorbable sutures.

456 Splenic denervation was achieved using ethanol, similarly to previous studies (Zhang et
457 al., 2020). In brief, an incision was made in the left abdomen as for splenectomy, and then the
458 spleen was isolated from the abdominal cavity using blunt forceps, while cotton pads moistened
459 with physiological saline were used to protect the abdominal opening and organs. Splenic
460 denervation was achieved by applying a cotton swab dipped in absolute ethanol to the vascular
461 trees and corresponding nerve fibers, approximately 7 times, 5-10 seconds per application. For
462 sham denervation the cotton swab was instead dipped in physiological saline solution (0.9% NaCl).
463 Finally, the spleen was returned to the abdominal cavity and incisions were closed as for
464 splenectomies.

465 Laser injury and bright light challenge

466 Laser injury and bright light challenge were performed as previously described (Roubeix
467 et al., 2024). For laser injury, mice received eye drops of mydriaticum and neosynephrine to dilate
468 the pupils, and then were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (8
469 mg/kg). Lubrithal was placed on the eye to maintain ocular surface moisture. For
470 immunohistochemistry 4 laser injuries were performed per eye in the mid-periphery using a
471 photocoagulation laser (Vitra Laser, 532 nm, 450 mW, 50 ms, 250 mm). For single-cell RNA
472 sequencing and flow cytometry experiments, 10 laser injuries were performed per eye. For bright
473 light challenge, the *TRE2* mice received daily eyedrops of atropine (1%, Novartis) to dilate their
474 pupils, and were exposed to green LED light (4500 lux) for 4 days.

475 Immunohistochemistry

476 Eyes were fixed in 4% PFA at room temperature (RT) for 45 minutes before storage in
477 PBS and dissection. Anterior segments and lens were removed, and after 4 curvature-relieving cuts
478 were made the retinas and RPE/choroid/sclera were carefully separated and then incubated
479 overnight at RT in PBS with 1% Triton X-100 with the following primary antibodies (dilutions
480 indicated in parentheses): rabbit anti-IBA1 (Wako, 1:400) and rat anti-CD102 (BD Biosciences,
481 1:200). After 3 PBS rinses, retinas and RPE/choroid/sclera complexes were incubated for 2 hours
482 at RT with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific, diluted 1:500)
483 and Hoechst (Sigma Aldrich, diluted 1:1000) in PBS. After 3 more PBS rinses, tissues were
484 flatmounted and coverslipped with an aqueous mounting medium (Sigma Aldrich) and imaged
485 using a fluorescence microscope (Leica, DM5500). Quantifications of MP counts and CNV size

486 were completed in FIJI. MPs and CNV size were quantified for individual laser impacts and were
487 then averaged by eye for further statistical analyses and graphical representation.
488 For spleen cryosections, spleens were flash frozen in liquid nitrogen, embedded in Neg-50
489 optimal cutting temperature compound (Fischer Scientific), and then 12 µm sections were cut on
490 a Leica CM3050 cryostat. Sections were fixed on the slide with 4% PFA for 15 minutes at RT,
491 blocked for 1 hour at RT in blocking solution (PBS + 0.1% Triton X-100 + 5% normal horse
492 serum), and then incubated overnight at 4 C with rabbit anti-tyrosine hydroxylase (Sigma-Aldrich,
493 1:200) and human anti-mouse CD3 (REAfinity, PE-conjugated, Miltenyi, 1:100) diluted in
494 blocking solution. After 3 PBS rinses, sections were incubated for 2 hours at RT with Alexa Fluor-
495 conjugated secondary antibodies (Thermo Fisher Scientific, 1:500) and Hoechst (Sigma Aldrich,
496 1:1000) in blocking solution. After 3 more PBS rinses slides were coverslipped with an aqueous
497 mounting medium (Sigma Aldrich) and imaged using a fluorescence microscope (Leica,
498 DM5500).

499 Galantamine treatment

500 Galantamine, an acetylcholinesterase inhibitor (Sigma Aldrich), was resuspended in
501 physiological 0.9% NaCl solution at a concentration of 1 mg/ml and then mice were given daily
502 IP injections of Galantamine (4 mg/kg) or an equal volume of saline for control.

503 Flow cytometry and fluorescence-activated cell sorting

504 Flow cytometry of eyes and spleens was performed as previously described (Roubeix et
505 al., 2024). In brief, spleens were mechanically dissociated on a 70-µm cell strainer (Miltenyi), red
506 blood cells were lysed using ammonium chloride (StemCell Technologies), and after rinsing in
507 PBS and centrifugation (500 g, 5 min, 4 C), cells were resuspended in PBS. Eyes were dissected
508 to remove posterior connective tissue from the sclera, the anterior segment, and the lens, and the
509 resulting posterior segment eye cups were enzymatically dissociated using Liberase TL in PBS
510 (0.8 Wunsch unit/ml, Sigma-Aldrich) followed by gentle mechanical dissociation using a P1000
511 pipette. The resulting cell suspension was passed through a 70-µm filter, rinsed with PBS, and
512 resuspended in PBS following centrifugation (500 g, 5 min, 4 C). Cells were labeled using a
513 live/dead fixable reagent (Miltenyi) following manufacturer recommendations, then incubated
514 with the relevant antibodies (all diluted 1/50 in PBS) for 30 minutes at 4 C. The antibodies used in
515 this study include: anti-CD45 VioBlue, anti-CD11b PE, anti-Ly6C PE-Vio770, anti-Ly6G APC-
516 Vio770, anti-CD3 FITC, anti-CD8a PE, and anti-CD4 PE-Vio770 (all from Miltenyi). For flow

517 cytometry, cells were fixed in 1% PFA, data acquisition was performed on a Celesta SORP
518 cytometer (BD Biosciences), and data were analyzed with FlowJo 10.9. For cell sorting, cells
519 suspensions were prepared as described for flow cytometry but without the final fixation step.
520 Cells were sorted into chilled tubes using a FACSMelody cell sorter (BD Biosciences).

521 Bulk mRNA sequencing

522 $CD11b^{+}CD45^{+}Ly6C^{neg}Ly6C^{high}$ splenic monocytes were sorted, as described above. RNA
523 was extracted and RNA quality was confirmed using a Bioanalyzer RNA Analysis kit (Agilent).
524 RNA sequencing libraries were prepared from 2 ng total of RNA using the Smartseq V4 (Takara)
525 and Nextera XT (Illumina) kits and sequenced at the Institut du Cerveau iGenSeq sequencing
526 facility.

527 After sequencing, fastq files were aligned to the *Mus musculus* reference genome from
528 Ensembl (Ensemble 106, June 2022) using STAR (version 2.7.9a) with the option "- - quantMode
529 Gene Counts" to extract the raw counts for each gene, and all count files were concatenated into a
530 single file. The count file and descriptive sample file were loaded into our in-house R Shiny
531 application "EyeDVseq" for analysis: the genes with a total count below 10 across samples were
532 filtered out and the differential gene expression analysis was performed using DESeq version
533 1.40.2 integrated into EyeDVseq, comparing sham as control and vagotomy as the condition. The
534 resulting gene list was then filtered, with $|\log_2(\text{FoldChange})| > 0.5$ and pAdj values < 0.05 being
535 considered significant.

536 Single-cell mRNA sequencing and analysis

537 Live $CD11b^{+}CD45^{+}$ MPs were sorted from eyecups with and without laser, as described
538 above. Single cell mRNA sequencing (scRNAseq) libraries were prepared from sorted cells using
539 the Chromium Next GEM Single Cell 3' Library v3.1 and dual index TT kits (10X Genomics)
540 according to manufacturer's recommended protocol. Resulting libraries were sequenced on an
541 Illumina Novaseq 6000. The fastq files were processed using CellRanger v6.1.2 with default
542 options (alignment, count). The resulting expression matrices were merged and analyzed with
543 Seurat v4 (merging, QC filtering, normalization, PCA, clustering, and UMAP). The resulting
544 Seurat object was used to create an H5AD file that was loaded into cellxgene. Further analyses,
545 including differential gene expression analyses, and visualizations were performed in cellxgene
546 using the cellxgene_VIP plugin. Cells other than MPs (including rod photoreceptors and other
547 retinal neurons) were excluded and then the remaining MPs were re-clustered. Differentially

548 expressed genes were identified using the cellxgene Welch's t test, and the resulting gene lists
549 were filtered with adjusted p-value < 0.05 and |log Fold Change| > 0.2 considered significant.
550 Processing scripts are available on request.

551 To examine gene expression in ocular macrophages from human AMD donor eyes, a
552 previously described and processed scRNAseq dataset from human choroids was downloaded with
553 the accession number GSE183320 (Voigt et al., 2022). This dataset was generated from 10 control
554 donor choroids and 9 donor choroids from eyes with intermediate or dry AMD. The data was
555 loaded into cellxgene and then the resident macrophage and infiltrating monocyte/macrophage
556 clusters from control and non-exudative AMD donors were extracted. Further analyses and
557 visualizations were performed using the cellxgene_VIP plugin, as described for the mouse MP
558 dataset, above.

559 Statistical analysis

560 GraphPad Prism 10 was used for data analysis and graphic representation, unless otherwise
561 stated (see above for details of the cohort study and RNA sequencing analyses). Data are presented
562 as mean ± standard error of the mean (SEM). Differences between two groups were analyzed using
563 an unpaired Mann-Whitney test and differences between multiple groups were analyzed using a
564 one-way ANOVA followed by a Tukey's multiple comparisons test. The n and p-values are
565 indicated in the figure legends.

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742 **Table 1.** Incidence rates and hazard ratios of age-related macular degeneration for
743 vagotomy patients surviving 10 years after vagotomy and their comparison cohort. AMD = age-
744 related macular degeneration; COPD = chronic obstructive pulmonary disease; CCI = Charlson
745 comorbidity index.

	No. of AMD	Incidence Rate (per 1000 person-years at risk) (95% CI)	Hazard ratio controlled for age and gender by design	Adjusted for age, gender and COPD	Adjusted for age, gender and Obesity related diseases	Adjusted for age, gender and Hypertension	Adjusted for age, gender and CCI
Comparison cohort	2911	2.30 (2.21-2.38)					
All vagotomy	368	2.94 (2.65-3.25)	1.38 (1.23-1.55)	1.37 (1.22-1.54)	1.38 (1.22-1.55)	1.38 (1.23-1.55)	1.36 (1.21-1.53)
Comparison cohort	1306	2.74 (2.59-2.89)					
Truncal vagotomy	174	3.82 (3.27-4.40)	1.47 (1.24-1.75)	1.47 (1.24-1.75)	1.47 (1.24-1.75)	1.47 (1.24-1.75)	1.45 (1.22-1.72)
Comparison cohort	1494	2.01 (1.90-2.11)					
Superselective vagotomy	182	2.43 (2.09-2.79)	1.30 (1.11-1.54)	1.29 (1.09-1.52)	1.30 (1.10-1.53)	1.30 (1.11-1.54)	1.29 (1.10-1.53)
Superselective vagotomy	182	2.43 (2.09-2.79)					
Truncal vagotomy	174	3.82 (3.27-4.40)	1.20 (0.97-1.48)	1.20 (0.97-1.48)	1.19 (0.96-1.48)	1.20 (0.97-1.48)	1.19 (0.96-1.48)

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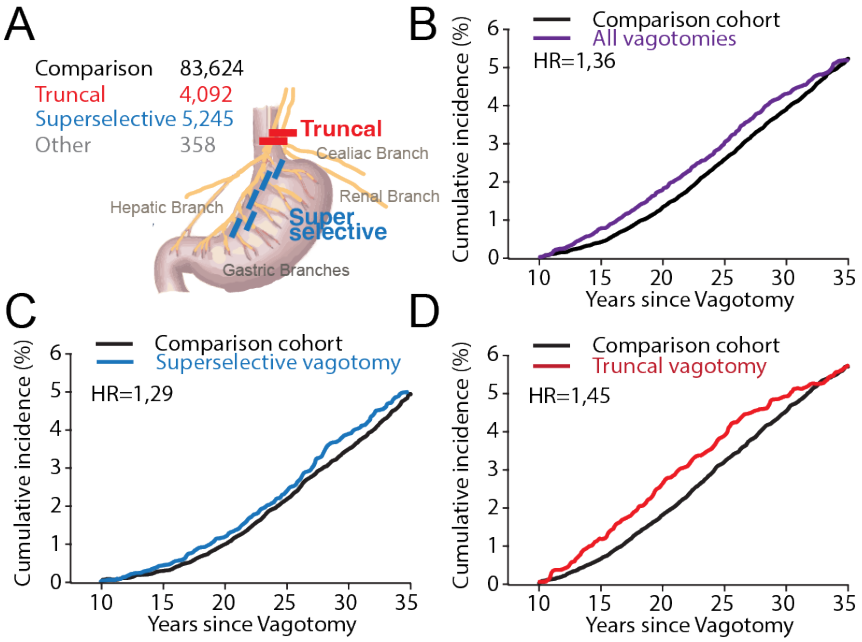

Figure 1. Vagotomy is associated with increased incidence of AMD.

(A) Study cohorts included all patients in Denmark who underwent vagotomy between 1977 and 1995 with ≥10 years follow-up (n=9,695), subdivided into truncal vagotomy (TV, n=4,092), superselective vagotomy (SSV, n=5,245), and unspecified type (n=358). Each patient was matched to 10 individuals from the general population (n=83,624) by exact birth year and sex using the Civil Registration System. The schematic illustrates that TV denervates abdominal organs including the coeliac branch that innervates the spleen, whereas SSV primarily targets gastric branches and spares extra-gastric innervation.

(B) Cumulative incidence of AMD in all vagotomized patients compared to their matched population cohort.

(C) Cumulative incidence of AMD in patients with superselective vagotomy compared to their matched cohort.

(D) Cumulative incidence of AMD in patients with truncal vagotomy compared to their matched cohort.

Follow-up began 10 years after index date to account for early differential mortality; curves were estimated using Kaplan–Meier analysis treating death as a competing risk. Hazard ratios (HR) were obtained from Cox regression adjusted for age, sex, COPD, obesity-related diseases, hypertension, and Charlson Comorbidity Index categories (full details in Table 1 and Supplemental Tables 1–3).

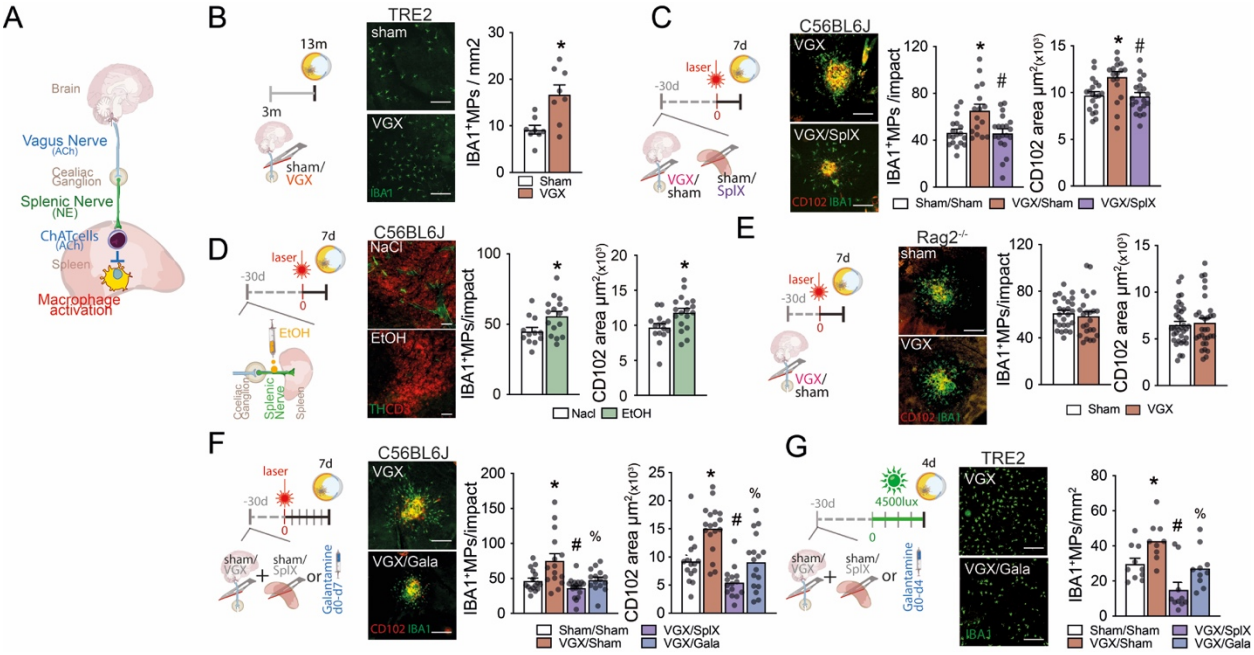

Figure 2. Vagotomy promotes inflammation and exacerbates disease severity in mouse models of AMD in a spleen-dependent manner.

(A) Schematic of the parasympathetic vagus–spleen axis: Acetylcholine from vagal efferents activate the splenic nerve at the coeliac ganglion, increasing splenic norepinephrine and driving ChAT^{+} T cell–derived acetylcholine that tonically restrains mononuclear phagocytes (MPs).

(B) Representative images and quantification of subretinal IBA1^{+} MPs on IBA1-stained RPE/choroidal flatmounts from TRE2 mice subjected to left cervical truncal vagotomy at 3 months and analyzed at 13 months (n=8 per group; Mann-Whitney test *p=0.0148).

(C) Laser-induced choroidal neovascularization (CNV) model in 3-month-old male C57BL/6J mice: vagotomy with or without splenectomy performed 1 month before laser (at 2 months old); quantification of subretinal IBA1^{+} MP counts and CD102^{+} CNV area on IBA1/CD102 double-stained RPE/choroidal flatmounts 7 days after laser injury (n= 16-20 per group; one-way ANOVA/Tukey: IBA1 Sham/Sham versus VGX/Sham *p=0.0057, VGX/Sham versus VGX/SplX #p=0.0034, Sham/Sham versus VGX/SplX not significant (ns); CD102 Sham/Sham versus VGX/Sham *p=0.0123, VGX/Sham versus VGX/SplX #p=0.0068, Sham/Sham versus VGX/SplX ns).

(D) Splenic denervation in 2-month-old male C57BL/6J mice by ethanol application at the splenic hiatus: (left) verification by loss of tyrosine-hydroxylase (TH, green)(co-stained for CD3,

787 magenta, and DAPI, blue) immunostaining on spleen sections; (right) quantification of subretinal
788 IBA1^{+} MP counts and CD102^{+} CNV area on IBA1/CD102 double-stained RPE/choroidal
789 flatmounts 7 days after laser injury (laser 1 month post-surgery)(n= 12-18 per group; Mann-
790 Whitney: IBA1 *p=0.0284; CD102 *p=0.0118).

791 (E) Laser model in 2-month-old male Rag2$^{-/-}$ mice (lacking mature lymphocytes including
792 ChAT^{+} T cells): quantification of subretinal IBA1^{+} MPs and CD102^{+} CNV area on IBA1/CD102
793 double-stained RPE/choroidal flatmounts 7 days after laser injury (n= 24-26 per group; Mann-
794 Whitney: IBA1 and CD102 not statistically significant).

795 (F) Daily intraperitoneal galantamine (an acetylcholinesterase inhibitor, 4 mg/kg) initiated
796 after laser in previously vagotomized 3-month-old male C57BL/6J mice: quantification of IBA1^{+}
797 MP counts and CD102^{+} CNV area on IBA1/CD102 double-stained RPE/choroidal flatmounts 7
798 days after laser injury (n= 15-17 per group; one-way ANOVA/Tukey: IBA1 Sham/Sham versus
799 VGX/Sham *p=0.005, VGX/Sham versus VGX/SplX #p<0.001, VGX/Sham versus VGX/Gala
800 %p=0.0093; CD102 Sham/Sham versus VGX/Sham *p=0.0009, VGX/Sham versus VGX/SplX
801 #p<0.0001, VGX/Sham versus VGX/Gala %p=0.0004).

802 (G) Bright-light challenge calibrated to induce subretinal MP infiltration in TRE2 but not
803 controls; quantification of IBA1^{+} MPs on IBA1-stained RPE flatmounts from 3-month-old male
804 TRE2 mice after 4 days of light exposure, one month after sham or vagotomy with or without
805 splenectomy or daily galantamine (4 mg/kg)(n=9-10 per group; one-way ANOVA/Tukey:
806 Sham/Sham versus VGX/Sham *p=0.0344, VGX/Sham versus VGX/SplX #p<0.0001,
807 VGX/Sham versus VGX/Gala %p=0.0285).

808 Statistics: data are represented as mean ± SEM, Mann-Whitney test (two-group
809 comparisons) or one-way ANOVA with Tukey's test (multiple comparisons) as indicated;
810 replicates represent quantifications of eyes or plasma samples from different mice (C57BL/6Js
811 were all males, TRE2 were males and females) from at least three independent cages/experiments.
812 ChAT: choline acetyltransferase; TRE2: targeted-replacement mice expressing human APOE2;
813 MP: mononuclear phagocyte; CNV: choroidal neovascularization. Scale bars: 20µm

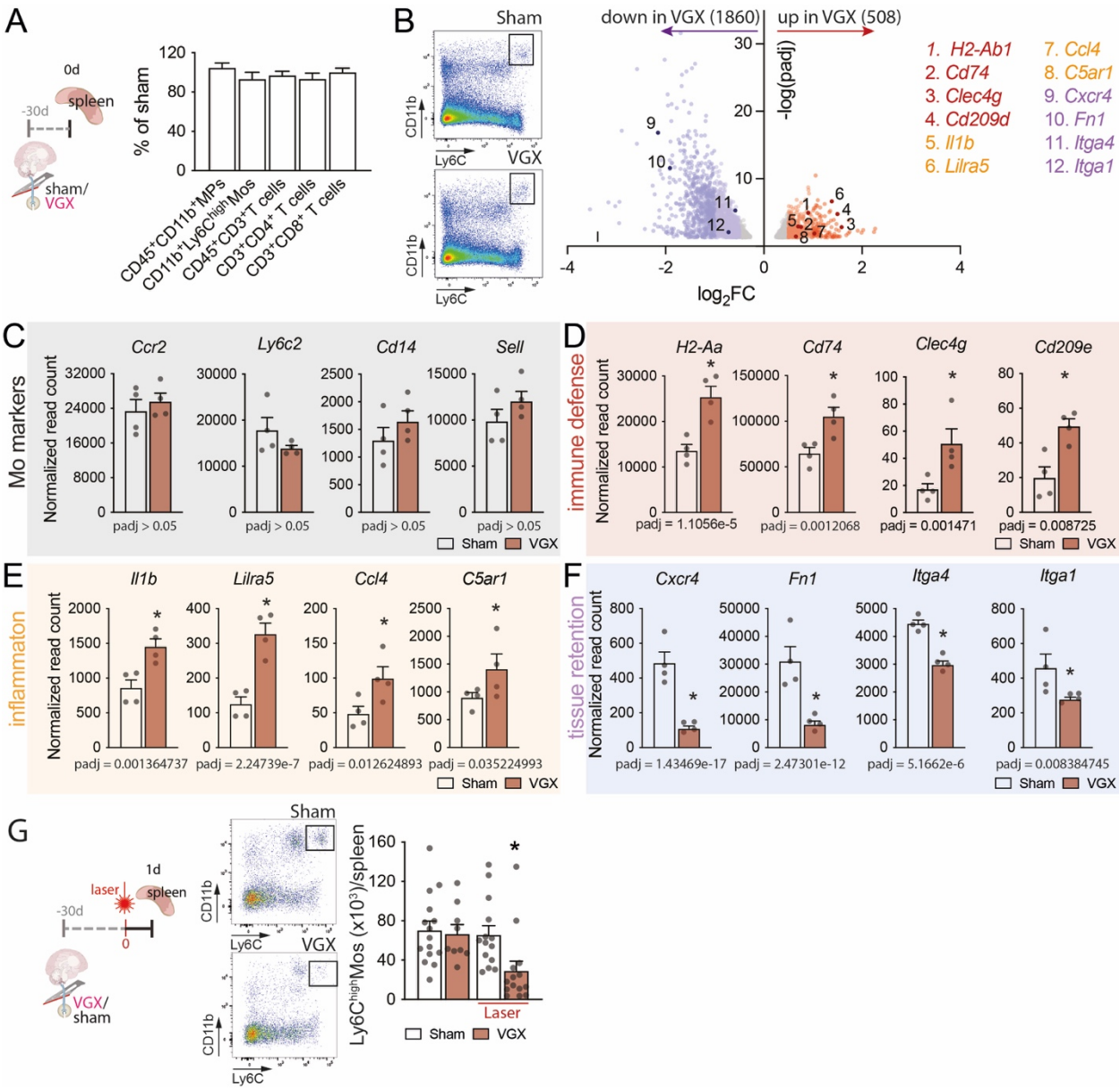

Figure 3. Loss of vagal tone reshapes the splenic monocyte transcriptome and drives their mobilization.

(A) Flow cytometric quantification of splenic CD45$^+$CD11b$^+$ MPs, CD45$^+$CD11b$^+$Ly6C^{high} monocytes (Mos), CD4$^+$ T cells, and CD8$^+$ T cells from 3-month-old male mice 30 days after vagotomy (VGX) or sham surgery, expressed as percentage of sham (see Supplemental Fig. 1 for comparisons between sham and VGX).

B) Bulk RNA sequencing of FACS-sorted CD45$^+$CD11b$^+$Ly6G^{neg}Ly6C^{high} splenic Mos: (left) representative flow plots; (right) volcano plot showing downregulated (n=1860) and upregulated (n=508) genes in VGX versus sham (right), with selected genes highlighted.

824 (C–F) Normalized read counts of selected genes: (C) classical monocyte markers (*Ccr2*,
825 *Ly6c2*, *Cd14*, *Sell*); (D) immune defense–associated genes (*H2-Aa*, *Cd74*, *Clec4g*, *Cd209e*); (E)
826 inflammatory genes (*Il1b*, *Lilra5*, *Ccl4*, *C5ar1*); (F) tissue retention–associated genes (*Cxcr4*, *Fn1*,
827 *Itga4*, *Itga1*). Adjusted p values are indicated in the figure.
828 (G) Experimental schematic of vagotomy/sham followed by laser injury or no injury 30
829 days later, with analysis at day 1 (left) and representative flow cytometric plots and quantification
830 of splenic Ly6C^{high} Mos from sham and VGX mice with or without laser (right, n=9-15 per group;
831 Mann-Whitney: Laser Sham versus VGX *p=0.001; no laser Sham versus VGX not statistically
832 significant).
833 Statistics: (G) data are represented as mean ± SEM, Mann-Whitney test (two-group
834 comparisons); (B-F) see methods for RNAseq analysis details; replicates represent spleens from
835 different mice (all males). MP: mononuclear phagocyte; Mo: monocyte; VGX: vagotomy.

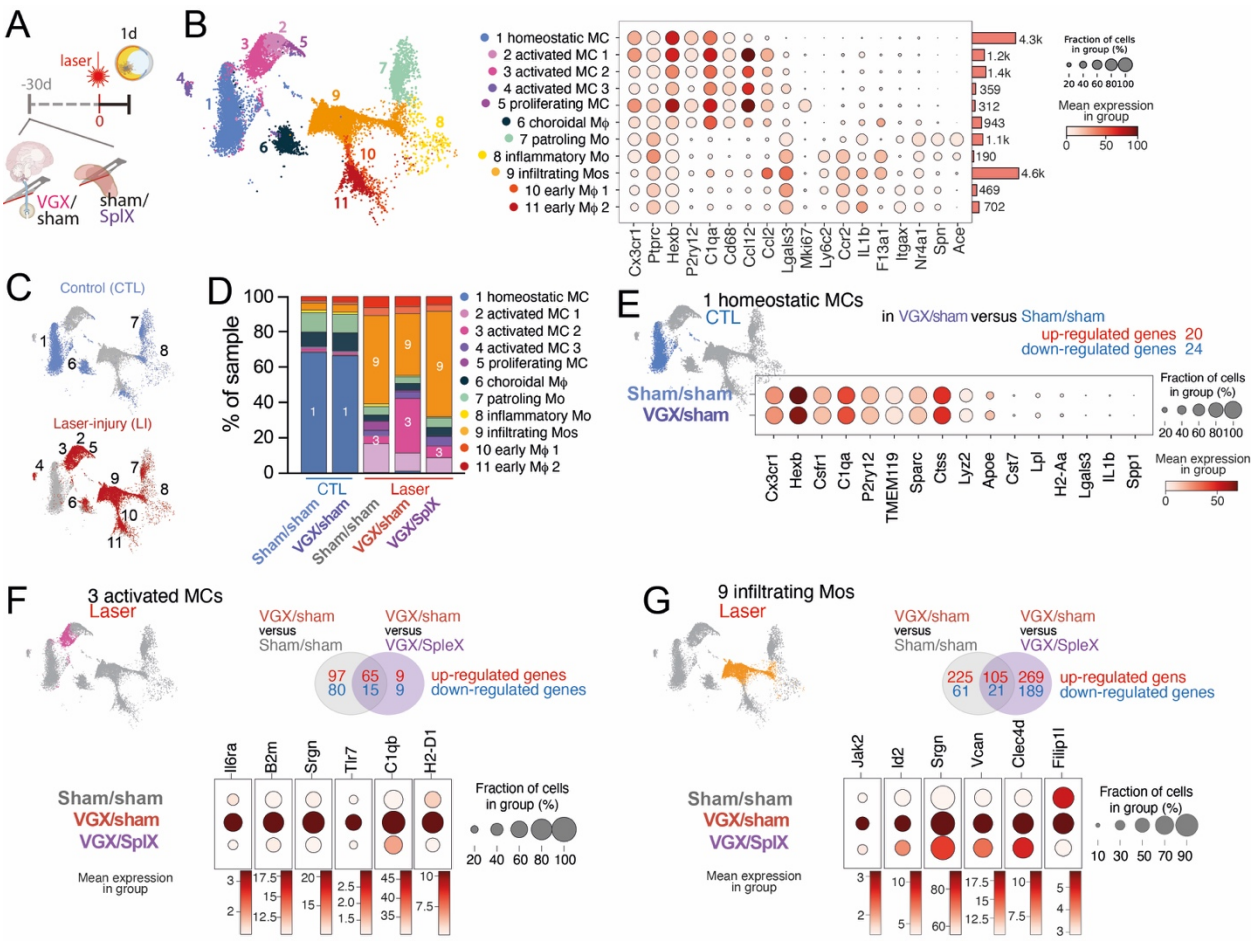

Figure 4. Vagotomy induces transcriptional reprogramming of infiltrating monocytes and activated microglia in the retina in a spleen-dependent manner.

(A) CD45^{+}CD11b^{+} cells were FACS-sorted from posterior eye segments of mice 1 month after sham or vagotomy (VGX), and from mice 1 day after laser injury that had undergone sham, VGX, or VGX with splenectomy (VGX+SPLX) one month prior.

(B) UMAP projection of myeloid cell clusters (left) and dot plot showing expression of representative marker genes (right).

(C) Distribution of cells between control (non-lasered, CTL) and lasered samples.

(D) Relative proportions of each cluster across experimental conditions.

(E) Expression of representative homeostatic and activated microglia genes in the homeostatic microglia cluster from non-lasered sham and VGX samples.

(F) Differential gene expression analysis of cluster 3 (activated MC2) between surgery conditions (top), with representative genes shown as dot plots (bottom).

850 (G) Differential gene expression analysis of cluster 9 (infiltrating monocytes, Mos)
851 between surgery conditions (top), with representative genes shown as dot plots (bottom).
852 VGX: vagotomy; SplX: splenectomy; CTL: control; LI: laser injury.