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Loss of Serpin E2 alters antimicrobial gene expression by microglia but not astrocytes

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Abstract

Microglia are the brain-resident immune cells responsible for surveilling and protecting the central nervous system. These cells can express a wide array of immune genes, and that expression can become highly dynamic in response to changes in the environment, such as traumatic injury or neurological disease. Though microglial immune responses are well studied, we still do not know many mechanisms and regulators underlying all the varied microglial responses. Serpin E2 is a serine protease inhibitor that acts on a wide variety of serine proteases, with particularly potent affinity for the blood clotting enzyme thrombin. In the brain, Serpin E2 is highly expressed by many cell types, especially glia, and loss of Serpin E2 leads to behavioral changes as well as deficits in synaptic plasticity. To determine whether Serpin E2 is important for maintaining homeostasis in glia, we performed RNA sequencing of microglia and astrocytes from Serpin E2-deficient mice in a healthy state or under immune activation due to lipopolysaccharide (LPS) injection. We found that microglia in Serpin E2-deficient mice had higher expression of antimicrobial genes, while astrocytes did not display any robust changes in transcription. Furthermore, the lack of Serpin E2 did not affect transcriptional responses to LPS in either microglia or astrocytes. Overall, we find that Serpin E2 is a regulator of antimicrobial genes in microglia.

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Keywords

microglia; Serpine2; astrocyte; antimicrobial; glia; inflammation

Introduction

As the resident immune cells of the brain, microglia play indispensable roles in immune surveillance and defense in the central nervous system. Accordingly, microglia can dynamically express a broad array of antimicrobial genes to initiate and mount an immune response. While we often refer to microglia as “activated” or “reactive” while undergoing an inflammatory response, there are myriad genes and pathways involved and which change based on the particular environmental stimuli[13, 25, 27, 33]. Given the underlying diversity of microglial responses to pathology, it is important to untangle these responses and identify underlying regulatory mechanisms that control specific portions of the immune response. While much is known about the innate immune functions including antimicrobial functions of microglia, the signals that regulate the expression of antimicrobial genes by microglia remain largely unidentified.

Serpin Family E Member 2 (Serpin E2, aka Protease-Nexin 1/PN1 or Glia-derived Nexin 1) is a serine protease inhibitor that can inhibit a number of enzymes[6, 8, 14, 16, 34]. In mice, it has particularly high RNA expression in the brain across most major cell types, including microglia, astrocytes, neurons, oligodendrocyte-lineage cells, and endothelial cells[40, 41]. Transgenic mice overexpressing Serpin E2 in neurons or lack Serpin E2 entirely develop epileptic activity *in vivo* and *in vitro*[21]. Overexpressing mice showed increases in long-term potentiation in the hippocampus, while deficient mice showed a reduction[21]. Serpin E2-deficient mice also showed a deficit in fear extinction after learning to associate a tone with a foot shock, as well as reduced availability of NR1 subunits in NMDA receptors, suggesting a potential NMDA-dependent mechanism underlying the behavioral changes[18, 23].

The mechanisms of Serpin E2 activity in the brain are complex and likely multifaceted. As Serpin E2 can act on several serine proteases, it may modulate the activity of many cellular signaling pathways [24]. Serpin E2 is a particularly potent inhibitor of thrombin, a key molecule in the formation of blood clots[34]. Thrombin also participates in other forms of signaling, including binding with the thrombin receptor PAR1. Experimental activation of PAR1 has been shown to induce amnesia and reduce LTP in hippocampal slices[15]. Other studies observed that the loss of Serpin E2 led to upregulation of Sonic hedgehog target genes *in vivo*[37]. In a cancer model, Serpin E2 may promote invasiveness via MMP9 and receptor LRP1 activity[9]. Taken together, Serpin E2 is a versatile protein with great potential to regulate a number of cellular processes.

Serpin E2-overexpressing mice under the Thy1 promoter demonstrate a functional role of neuronal Serpin E2, but the *Serpine2* gene is more highly expressed among glia[40, 41]. Whether and how Serpin E2 impacts glial cells is unknown. In this study, we use *Serpine2* deficient mice to examine the impact of Serpin E2 on the transcriptome of astrocyte and microglia in healthy and reactive states. We purified microglia and

astrocytes from *Serpine2* deficient and control mice undergoing inflammatory responses to lipopolysaccharide (LPS) injection and mice who received a control saline injection. Upon RNA sequencing and subsequent analysis, we found that loss of *Serpine2* results in the upregulation of numerous antimicrobial genes in microglia. Astrocytes did not show appreciable *Serpine2*-dependent changes in transcription, nor did *Serpine2* deficiency result in robust changes in LPS-induced gene expression. Overall, we identify Serpin E2 as a regulator of antimicrobial genes in microglia, without altering astrocyte transcription or glial responses to inflammatory stimuli.

Methods

Experimental animals

All animals were used in compliance with the Animal Research Committee at the University of California, Los Angeles (UCLA) under the approved protocol #R-16–080. We obtained *Serpine2*$^{-/-}$ mice from the lab of Dr. Thomas Mariani at the University of Rochester. We injected two cohorts of mice with 10 mg/kg lipopolysaccharides (LPS) or a control saline solution (Sigma L6529). Both cohorts consisted of 12 mice, where mice differed by genotype (*Serpine2*$^{+/+}$ vs. *Serpine2*$^{-/-}$) and treatment (LPS *vs.* saline), resulting in 4 treatment groups with 3 mice per group. The microglia cohort was approximately 1 month old and received IP injections 48 hours before purifying and harvesting microglia RNA. The astrocyte cohort was approximately 2 months old and received IP injections 24 hours before purifying and harvesting astrocyte RNA. Slightly different ages were used for microglia and astrocytes due to breeding constraints while targeting a similar young adult time point for both cell types. The microglia and astrocyte cohorts consisted of 7 males, 5 females and 8 males, 4 females respectively. 30- and 60-day old mice were also used to perform immunostaining of microglia, detailed under “Immunohistochemistry”.

Purification of microglia and astrocytes

We purified microglia and astrocytes using two distinct approaches. For microglia, we followed a previously published protocol using douncing and immunopanning that aimed to minimize microglial reactive response to the brain dissociation process[2]. Animals received transcardiac perfusions of ice-cold DPBS (Fisher 14–040-182) for 10 minutes to remove blood cells from the brain. After harvesting the whole brain, we removed olfactory bulbs before roughly chopping the tissue and douncing on ice in DPBS supplemented with DNase I (Worthington LS002007). To remove myelin debris, we brought the dounced cell suspension to 25 mL in a 20% Percoll solution (GE Healthcare 17–0891-01). We spun down the cell suspension for 15 minutes at 500 g and 4°C to isolate and remove myelin debris. Cells were resuspended in DPBS with 2 mg/mL milk peptone solids (Sigma P6838). The cell suspension was incubated for 20 minutes at room temperature on a plastic petri dish coated with anti-CD11b antibody to bind microglia (Biolegend 101202). The dish was washed with PBS and the cells were immediately scraped off the dish using 700 μL TRIzol (ThermoFisher 15596018), which was flash frozen using liquid nitrogen and stored at −80°C.

For astrocytes, we utilized our standard immunopanning protocol. Briefly, we dissected out the cerebral cortex as opposed to whole brain because we wanted to minimize myelin contamination; the cerebral cortex is a region that is large, easily dissected, and contains low levels of myelin. Next, we roughly chopped the tissue and incubated it for 45 minutes in a papain solution (12 u/mL, Worthington LS003126) while heated to 34.5°C. Digestion was halted using a trypsin ovomucoid inhibitor (Worthington LS003086), and tissue was further digested using mechanical trituration with a serological pipette. The resulting solution was passed through a Nitex filter and spun down at 300 g for 5 minutes. Cells were resuspended in DPBS and incubated on a series of antibody-coated petri dishes. The cell suspension was incubated on a series of dishes for 10 mins each: anti-CD45 ×2–3 to remove microglia (BD Biosciences 550539), anti-O4 hybridoma supernatant x2–3 to remove oligodendrocyte progenitors, and anti-GalC hybridoma supernatant x2–3 to remove oligodendrocytes. Finally, the astrocyte-enriched cell suspension was passed to an anti-HepaCAM dish to pull down astrocytes (R&D Systems MAB4108), and it was incubated at room temperature for 20 minutes. The dish was rinsed with PBS, and astrocytes were scraped off the dish with 700 μL TRIzol and immediately flash frozen in liquid nitrogen.

RNA-sequencing library construction and sequencing

RNA was purified from TRIzol lysates using the Qiagen miRNeasy kit (Qiagen 217004) according to the manufacturer's protocol. Purified RNA was converted to cDNA using the Ovation RNAseq System V2 (Nugen 7102–32), specifically designed for low input. We fragmented the cDNA using a Covaris S220 focused-ultrasonicator (Covaris 500217), and final libraries were constructed using the NEB Next Ultra RNA Library Prep Kit (New England Biolabs E7530S) along with appropriate indexing primers (NEB E7335S). Libraries from the same cell type were pooled and sequenced together on an Illumina NovaSeq 600 System to obtain paired-end 50 bp reads. The sequenced libraries had 40M ± 13M (s.d.) reads per microglia sample and 38M ± 8.7M reads per astrocyte sample.

Read alignment and quantification

Sequencing data was demultiplexed and aligned to the mouse genome (astrocytes: GRCm38, release 100, microglia: GRCm39, release 103) using the program STAR (astrocytes: v2.6.0c, microglia: 2.7.8a)[5]. Microglia samples had 80.0% ± 2.1 (s.d.) uniquely aligned reads, and astrocyte samples had 69.1% ± 6.6 uniquely aligned reads. After alignment, we obtained read counts using HTSeq v0.13.5 in RStudio[1]. Full RNA-seq expression data can be found in the supplemental information (S1).

Differential gene expression analysis with DESeq2

We performed differential gene expression (DGE) analysis in RStudio using the DESeq2 package (v1.26.0)[20]. For both astrocytes and microglia, we analyzed all samples from the same cell type together using a linear model that included terms for genotype (wild-type or *Serpine2*$^{-/-}$) and treatment (LPS or saline). For microglia, we also included a term for sex (male or female) and RNA integrity, as measured by the 2200 TapeStation System (Agilent G2964AA) and the RNA high sensitivity assay (Agilent 5067–5579). The resulting

analyses of differentially expressed genes associated with genotype and LPS can be found in supplemental information (S2).

Gene set enrichment analysis (GSEA)

We performed gene set enrichment analysis using GSEA software version 4.2.3 downloaded from www.gsea-msigdb.org [36]. We used the default settings, with the following specifications. We input gene counts that were normalized using the estimateSizeFactors() and counts() functions from the DESeq2 package v1.26.0. Based on our DGE results, we compared saline-injected wild-type and mutant mice to find transcriptional patterns associated with Serpine2. We used all gene ontology (GO) datasets that were built into the GSEA software (c5.all.v7.5.1).

Data deposition

We are in the process of depositing all gene expression data to the Gene Expression Omnibus. Raw sequencing data can be made available upon request.

Immunohistochemistry

Mice received transcardial perfusions of PBS (10 mins) followed by 4% paraformaldehyde (10 mins). Brains were post-fixed in 4% PFA overnight at 4°C before being stored in 30% sucrose for 1–2 nights at 4°C until brains sank. Brains were embedded in OCT compound (Fisher Scientific 23–730-571) and sectioned at 30 μm thickness and stored as free-floating sections at 4°C prior to staining and mounting. For immunostaining, sections were blocked and permeabilized with a blocking solution (0.2% Triton X-100 and 10% donkey serum in PBS) for one hour at room temperature, and then slides were incubated in similar blocking solution with primary antibodies overnight at 4°C (0.5% Triton X-100 for Iba1, 0.05% Triton X-100 for P2ry12 and 10% donkey serum in PBS). The following day, primary antibodies were washed off with PBS, and sections were incubated with secondary antibodies in blocking solution for 90 minutes at room temperature. Slices were mounted onto Superfrost Plus slides (Fisher Scientific 12–550-15) and coverslipped with a DAPI-containing media. Primary antibodies: anti-Iba1 (1:200 AbCam ab5076), anti-P2ry12 (1:500 Anaspec AS-55043A); Secondary antibodies: Donkey anti-rabbit 488 (1:1000 Life Technologies A21206), dokey anti-goat 488 (1:1000 Life Technologies A11055).

Iba1 and P2ry12 staining was performed with 3 pairs of mice at P30 (2 males, 4 females) and 3 pairs of mice at P60 (3 males, 3 females), and images were acquired in the cerebral cortex using a confocal microscope. Signal intensity of Iba1 and P2ry12 was measured with the resulting images in ImageJ after masking with a manual binary threshold to eliminate background. We further quantified aspects of microglial morphology using images of P2ry12 staining in 3 pairs of mice at P60. Images were analyzed using the “filaments” function in Imaris software. Following automatic detection of processes, “over-detected” filaments that did not represent microglial processes were manually deleted. Measurements of process number and length were quantified and exported for statistical analysis.

Statistics

Statistical analyses for immunohistochemistry were performed using either R or Excel, using Welch's t-test. Statistical analysis of differential gene expression was carried out within the DESeq2 package, and gene set enrichment analysis was performed using GSEA software v4.3.2, as previously described.

Results

Transcriptome profiling of microglia and astrocytes from *Serpine2*$^{-/-}$ mice

To examine how the loss of *Serpine2* may impact the transcriptome of astrocytes and microglia, we obtained and bred a colony of *Serpine2* knockout mice. We bred sibling pairs of 1 month (microglia) or 2 month (astrocyte) old mice consisting of a *Serpine2*$^{-/-}$ mouse and a *Serpine2*$^{+/+}$ or *Serpine2*$^{+/-}$ littermate. Of the 6 pairs collected per cell type, half were injected with saline while the other half were given injections of lipopolysaccharides (LPS) to induce systemic inflammation. This allowed us to ask not only whether *Serpine2* impacts homeostatic transcription but also whether *Serpine2* is required for the robust inflammatory responses that microglia and astrocytes normally exhibit in response to LPS [28, 39].

We acutely harvested microglia through a combination of douncing and immunopanning (Fig 1A, top). Microglia can rapidly alter their transcription during cell purification protocols in a temperature dependent manner[22]. To combat technical artifacts, we performed most of the cell extraction over ice with pre-chilled reagents. Mice were perfused and dissected using ice-cold PBS. Then, tissue was dounced over ice, which results in the death of most brain cell types while preserving microglia. Myelin debris was removed by spinning the cell suspension down through a Percoll solution at 4°C. Finally, the cell suspension was passed over a petri dish coated with a microglia-binding antibody Cd11b. RNA was harvested from the bound microglia using TRIzol, which was followed by sequencing.

For astrocytes, we acutely purified cells using an immunopanning method (Fig 1A, bottom). We dissected out the cerebral cortex to exclude myelin rich regions and improve purification. Tissue was then enzymatically digested in papain before mechanical trituration to create a single-cell suspension. We passed the cell suspension over a series of antibody-coated petri dishes to remove unwanted cell types (microglia, oligodendrocyte progenitor cells, and oligodendrocytes), and we pulled down astrocytes on a dish covered with an astrocyte-binding antibody for HepaCAM. As with the microglia samples, RNA was harvested using TRIzol, purified, and sequenced for analysis. Upon sequencing we find the expected expression of *Serpine2* among microglia and astrocytes in wildtype mice (Fig 1_), which agrees with our previous RNAseq studies[40].

Serpine2$^{-/-}$ microglia upregulate antimicrobial genes

After sequencing, we performed differential gene expression analysis of *Serpine2*$^{-/-}$ microglia using the DESeq2 package in R. When comparing *Serpine2*$^{-/-}$ microglia to controls, we found numerous genes upregulated in *Serpine2*-deficient microglia, in addition to the highly significant downregulation of *Serpine2* itself. There were 12 protein-coding

genes differentially expressed, and all of them had higher transcription in *Serpine2*$^{-/-}$ compared to controls (Fig 2A, S2). Among these genes was *Lyz2*, which codes for an enzyme that damages bacterial cell walls to promote lysis and eliminate microbes[3, 11]. *Hp* is another upregulated gene that encodes a protein with antibacterial properties, haptoglobin. Haptoglobin binds hemoglobin and therefore acts as an iron scavenger that impairs bacterial survival[7]. *Seprine2*$^{-/-}$ microglia also increased transcription of *Lgals3*, which encodes Galectin 3, yet another protein with noted antimicrobial activity. One proposed mechanism of action is that Galectin 3 mediates the destruction of pathogen containing vacuoles where microbes sometimes propagate within a host cell[10]. *S100a8* encodes a protein that forms a heterodimer with S100A9 with antimicrobial properties named calprotectin[38]. *Camp* encodes cathelicidin antimicrobial peptide, which is a member of the cathelicidin family of proteins known primarily for their capacity to kill microbes[12, 31]. Lastly, *Serpine2*$^{-/-}$ mice also upregulate *Clec4e*, the gene encoding the protein Mincle which binds microbial ligands and helps initiate the activation of antigen presenting cells[32].

While examining differentially expressed genes individually, we clearly observed repeated annotations of antibacterial activity among these upregulated genes. To better describe this pattern with an unbiased approach, we performed Gene Set Enrichment Analysis (GSEA) [36]. GSEA uses all the expression data from control and knockout microglia to test whether various pathways are up- or down-regulated. We tested our data against a over 15,000 ontology gene sets, and we found that *Serpine2*$^{-/-}$ microglia show greater expression of 21 gene sets (FDR = 0.05), while no gene sets demonstrated higher expression in control microglia (Fig 2B, S3).

All the significant gene sets pertaining to biological processes concerned immune and antimicrobial responses, e.g. defense response to bacterium (Fig 2C), antibacterial humoral response, immune response in mucosa. This directly aligns with the results of our differential gene expression analysis, which also identified upregulation of antimicrobial genes in transgenic microglia. With the combined results of these analyses, we found that microglia have increased transcription of antimicrobial genes in the absence of Serpin E2.

Microglia are known to make distinct morphological changes when they are reactive, such as thicker and shorter processes[29]. To test whether changes in immune response genes we observed had corresponding changes in morphology, we performed immunostaining of canonical microglial markers Iba1 and P2ry12 (Fig 3A,C). We found no difference in the average intensity or area of Iba1 staining ($p = 0.95$) or P2ry12 staining ($p = 0.87$, Fig 3B&D), which typically change when microglia enter reactive states. We also failed to find a change in microglia density, which we might observe if microglia became activated and proliferative (Fig 3B). Since P2ry12 antibody staining intensity appeared normal and provided high signal with low background, we used P2ry12 staining to quantify aspects of microglial morphology, namely branch number ($p = 0.51$) and branch length ($p = 0.88$, Fig 3D). None of these measures showed differences in transgenic mice compared to controls, indicating that *Serpine2* is dispensable for microglial morphology.

We also note that both male and female mice were used for microglia and astrocyte sequencing to maximize the generalizability of our findings. Due to known sex differences

in microglia, we used sex as a covariate in our differential gene expression analysis. However, we do not present an analysis of gene expression associated with sex because our experimental design sometimes resulted in a single male or female in a particular treatment group, and it would be inappropriate to extrapolate information about sex from individual mice.

Astrocyte transcriptome is unchanged in the absence of *Serpine2*

Using a similar methodology, we also employed DESeq2 to identify differential gene expression in astrocytes from *Serpine2*$^{-/-}$ vs. control mice. In this comparison, there was only one gene that showed differential expression, aside from the highly significant downregulation of *Serpine2* (Fig 1A). However, this gene, *Scg2*, is only lowly expressed in astrocytes and much more highly expressed in neurons[40, 41]. Therefore, we did not find evidence that astrocytes alter their transcriptomes in the absence of Serpin E2. This is somewhat surprising, given that we previously found astrocytes highly express *Serpine2*[40, 41].

Microglia and astrocytes respond robustly to LPS in the absence of *Serpine2*

Microglia and astrocytes are two important classes of glia that are known for their abilities to dynamically change state in response to environmental stimuli. This is a well-studied phenomenon variously referred to as “activation” or “reactivity”[29]. Whether or not Serpin E2 played an important role in these cells during homeostasis, it is also critical to know whether it modulates their responses to pathological conditions. To test this question, we induced a systemic inflammatory response in *Serpine2*$^{-/-}$ and control animals with injections of lipopolysaccharides, which are major surface components of many bacteria that trigger a robust response via binding to toll-like receptor 4 (TLR4) expressed by microglia in mice. Microglia and astrocytes both responded robustly to LPS exposure in wildtype and mutant mice, which both demonstrated glia with more amoeboid-like morphology. We observed increased soma size in microglia (Fig 4A) and loss of fine processes in astrocytes (Fig 4B).

Differential gene expression analysis found that LPS successfully induced large transcriptomic changes in both astrocytes and microglia, as expected (Fig 1A). Microglia had over 800 differentially expressed gene entries, while astrocytes had over 2,000. This response broadly agrees with previous RNA-seq analyses of microglia and astrocytes, which find hundreds to thousands of differentially expressed genes after LPS exposure [4, 28]. LPS efficacy is highly batch and dose dependent, so some differences in effect size are to be expected. However, when we looked among LPS-treated animals and compared *Serpine2*$^{-/-}$ to controls, we saw virtually no evidence that Serpin E2 regulates glial responses to LPS. LPS-treated astrocytes showed no differential expression associated with genotype, other than *Serpine2* itself. LPS-treated microglia showed a short list of genes associated with genotype, though only three entries were protein-coding and all had very low levels of gene expression (S2). Additionally, none of these genes were among the genes that were associated with genotype in our previous analysis of microglia. Taken together, we find that the loss of *Serpine2* can alter gene expression by microglia in the healthy brain, but it does

not change microglial or astrocytic responses to inflammatory stimuli, at least under the conditions we tested.

Discussion

We collected and analyzed transcriptomic data from mice lacking the gene for the serine protease Serpin E2. We examined both microglia and astrocytes, two major populations of glia with appreciable expression of this gene. Through differential gene expression analysis, we found that microglia increase the expression of numerous antimicrobial genes, and we further confirmed this pattern in a follow-up analysis using GSEA. Gross microglial morphology did not accompany these changes. Despite our previous observation that *Serpine2* was expressed highly in astrocytes, *Serpine2*$^{-/-}$ astrocytes did not change in gene expression[17, 40, 41]. Furthermore, astrocytes and microglia could both become reactive in response to an inflammatory stimulus, but the presence or absence of *Serpine2* did not affect these responses. Taken together, these findings demonstrate that *Serpine2* expression is a regulator of antimicrobial genes in microglia in the healthy brain, but not an important regulator of astrocyte transcription or glial responses to inflammatory stimuli.

Microglia in *Serpine2*$^{-/-}$ mice

In our previous studies, microglia appeared to have lower *Serpine2* expression when compared with astrocytes in both mouse and human tissue[40, 41]. Interestingly, this study finds that microglia do in fact upregulate numerous genes in the absence of *Serpine2*. A clear majority of them are involved in immune responses, particularly antimicrobial responses (*Lyz2*, *Hp*, *Lgals3, S100a8, Camp, Clec4e*). It is important to note that we performed *in situ* hybridization with probes for four differentially expressed genes but failed to validate our RNAseq results. However, the larger pattern of gene expression was further demonstrated by GSEA, which found a number of immune response pathways upregulated in *Serpine2*$^{-/-}$ microglia (defense response to bacterium, antibacterial humoral response, etc.) based on the entire transcriptome. These transcriptome-wide gene signatures provides support to our general conclusions, though claims about individual genes require further investigation. Based on these data, we conclude that *Serpine2* is a regulator of antimicrobial genes in microglia. Still, the mechanism by which *Serpine2* alters antimicrobial gene expression remains unclear. Serpin E2 in a potent inhibitor of several serine proteases, including thrombin, trypsin, and plasminogen activators. Tissue-type plasminogen activator (tPA) is involved in generating microglial inflammatory responses, so removing its inhibitor Serpin E2 could logically increase expression of immune response genes. It is curious that loss of *Serpine2* impacts such a specific set of antimicrobial genes as opposed to other aspects of the immune response, but perhaps compensation of other serine protease inhibitors prevents larger aberrations. Loss of *Serpine2* may impact microglia more significantly in other contexts, such as cerebrovascular challenges that would likely result in altered thrombin and blood clotting activity. As always, assessing protein-level changes in *Serpine2*$^{-/-}$ mice is an important future direction as protein-level regulation often differs from RNA-level regulation.

In this current study, we detect no protein-level changes in *Serpine2*$^{-/-}$ microglia. Microglia did not change in morphology or expression of two major markers, P2ry12 and Iba1. This refutes the idea that these microglia are partially reactive, or "primed", despite the increased expression of several immune response genes. In fact, the presence of *Serpine2* did not affect the transcription of immune response genes in microglia after we induced systemic inflammation with LPS. Systemic inflammation resulted in large changes in microglial transcription, compared with a modest effect of *Serpine2* genotype. The overwhelming response to LPS may in fact mask small or moderate interactions with *Serpine2* which could come to light in larger studies. However, *Serpine2* is clearly not a necessary component of microglial LPS responses. If Serpin E2 plays a role in systemic inflammation, it is possible that role is redundant with other serpin family proteases. This kind of compensation is particularly common in full knockout models like the one used in the present study. Nevertheless, we have found that *Serpine2* is capable of exerting an influence on microglia through a mechanism that remains unclear. It is possible that *Serpine2* plays a more substantial role in regulating microglial responses in other contexts not explored in this study, such as development, where microglia play vital roles in refining neuronal circuitry[26, 30]. Indeed, some evidence already suggests that *Serpine2* affects neuronal progenitor proliferation in this epoch[19].

Astrocytes in *Serpine2*$^{-/-}$ mice

This study did not find evidence that astrocyte transcription is altered by the presence or absence of *Serpine2*. Given the high expression of this gene in our previous astrocyte studies, this came as a surprise[40, 41]. We also previously reported that *Serpine2* itself was dynamically expressed in human astrocytes in a tumor context, so we hypothesized that *Serpine2* might play some part in astrocyte reactivity[17]. Interestingly, there was no gene expression associated with the loss of *Serpine2*, even when astrocytes did show a robust response to an inflammatory stimulus.

This raises the question of the function of *Serpine2* transcription in astrocytes. Importantly, Serpin E2 is a secreted molecule and it is expressed by many cell types in the brain, so astrocytes may produce Serpin E2 for it to act elsewhere in the brain. As Serpin E2 is a potent thrombin inhibitor involved in the blood clotting pathway, and astrocytic endfeet line blood vessels of the brain, perhaps expression of *Serpine2* is a preemptory strategy for addressing potential brain bleeds[35]. It would be interesting for future studies to assess whether *Serpine2* expression in astrocytes plays a role in hemorrhagic stroke responses.

Overall, this present study elucidates aspects of microglial biology as well as providing further knowledge of a protein that is ubiquitously transcribed in the brain. Our findings show that *Serpine2* regulates the expression of antimicrobial genes in microglia. It also describes limits to the influence of *Serpine2*, which is important knowledge given the broad expression and myriad protein-protein interactions of the Serpin E2 protein in the brain. At the same time, we have uncovered a novel regulator of immune response genes in microglia, and these discoveries will in turn increase our knowledge of neuroimmune interactions and ultimately the roles of microglia in neuropathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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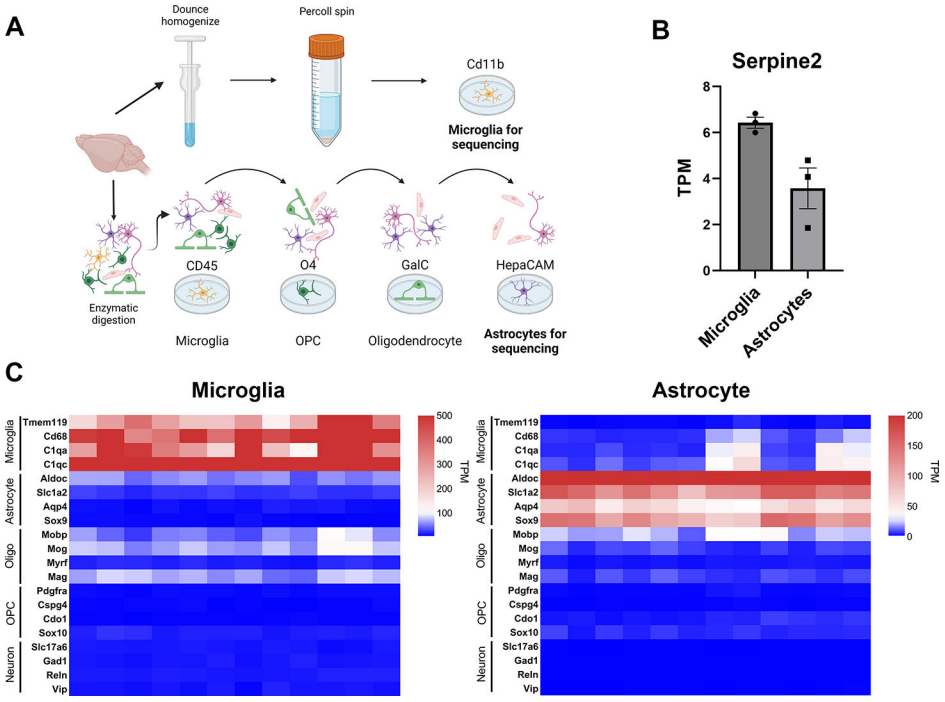

Fig 1. Acute purification of microglia and astrocytes from *Serpine2*$^{-/-}$ mice.

A) Cell purification schematic; top: microglia were collected with a combination of douncing and immunopanning with anti-Cd11b antibody, bottom: astrocytes were collected with an enzymatic digestion followed by immunopanning that depleted other cell types before binding astrocytes using anti-HepaCAM antibody. B) RNA expression of *Serpine2* in microglia and astrocytes of wildtype mice. C) Heatmap of brain cell-specific markers (rows) detected in immunopanning purified microglia (left) and astrocytes (right) as determined by RNA-sequencing; gene expression is quantified as Transcripts per Million (TPM).

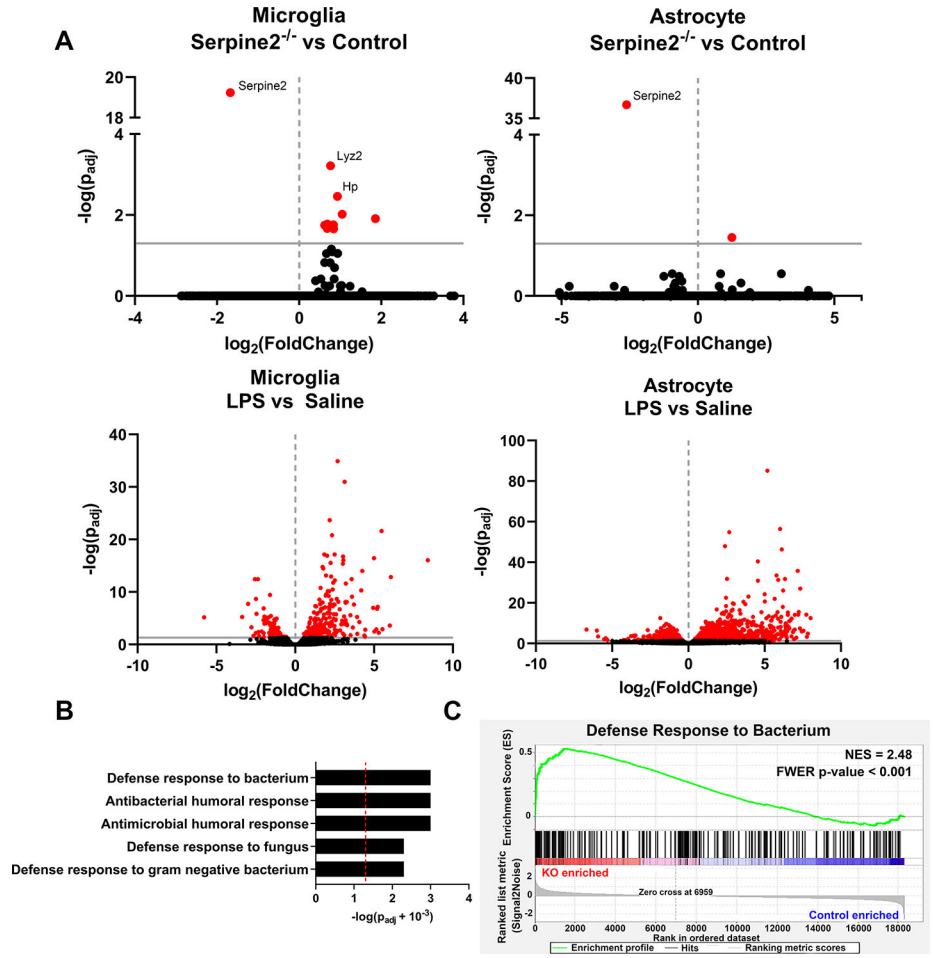

Figure 2. Transcriptomes of microglia and astrocytes in *Serpine2*$^{-/-}$ mice.

A) Volcano plots of the differential gene expression analysis results for microglia (top) and astrocytes (bottom) with respect to genotype (left) and LPS response (right). Red = p-adjusted < 0.05. B) Gene set enrichment analysis (GSEA) results for *Serpine2*$^{-/-}$ microglia, selected terms with significant enrichment. Dashed line marks p = 0.05 C) GSEA detailed results of one term significantly enriched in *Serpine2*$^{-/-}$ microglia; NES = normalized enrichment score, FWER = family-wise error rate.

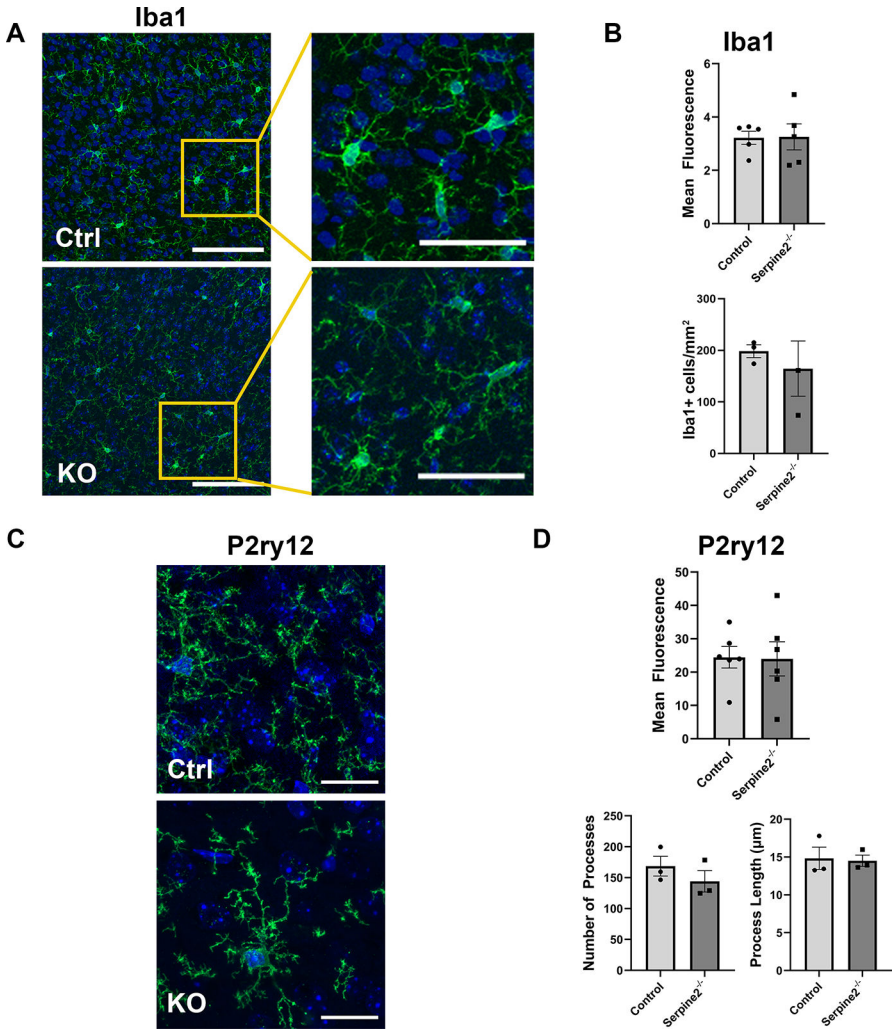

Figure 3. Microglial morphology in *Serpine2*$^{-/-}$ mice.

A) Example images of microglial marker Iba1 in *Serpine2*$^{-/-}$ and control mice and high magnification insets. Green = Iba1, blue = DAPI. Full image scale bar = 100 μm, inset scale bar = 50 μm. B) Quantification of Iba1 fluorescence (top) and microglia density (bottom) in *Serpine2*$^{-/-}$ vs. control animals. C) Example images of microglial marker P2ry12. Green = P2ry12, blue = DAPI. Scale bar = 20 μm. D) Quantification of P2ry12 fluorescence (top) and microglial process morphology (bottom) as analyzed with Imaris using images of P2ry12 fluorescence.

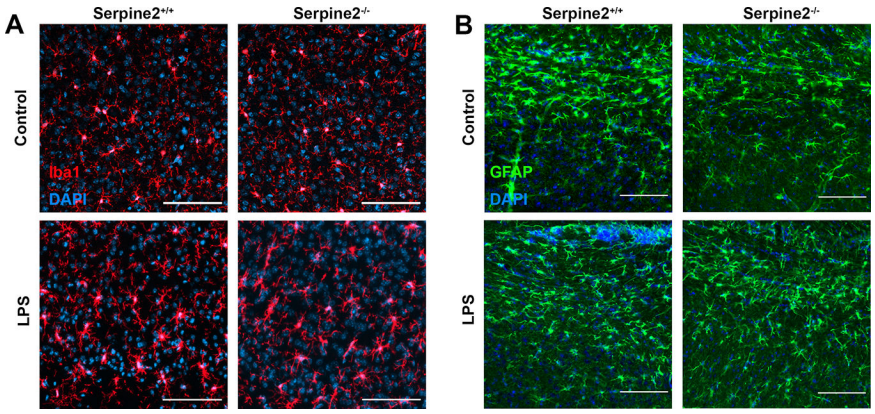

Figure 4. Morphology of glia after LPS-induced inflammation.

A) Microglia labelled with Iba1 in control (top) and LPS-injected (bottom) mice. B) Astrocytes from the corpus callosum labelled with GFAP in control (top) and LPS-injected (bottom) mice.