REPORT

Restoring periodontal tissue homoeostasis prevents cognitive decline by reducing the number of Serpina3n^{high} astrocytes in the hippocampus

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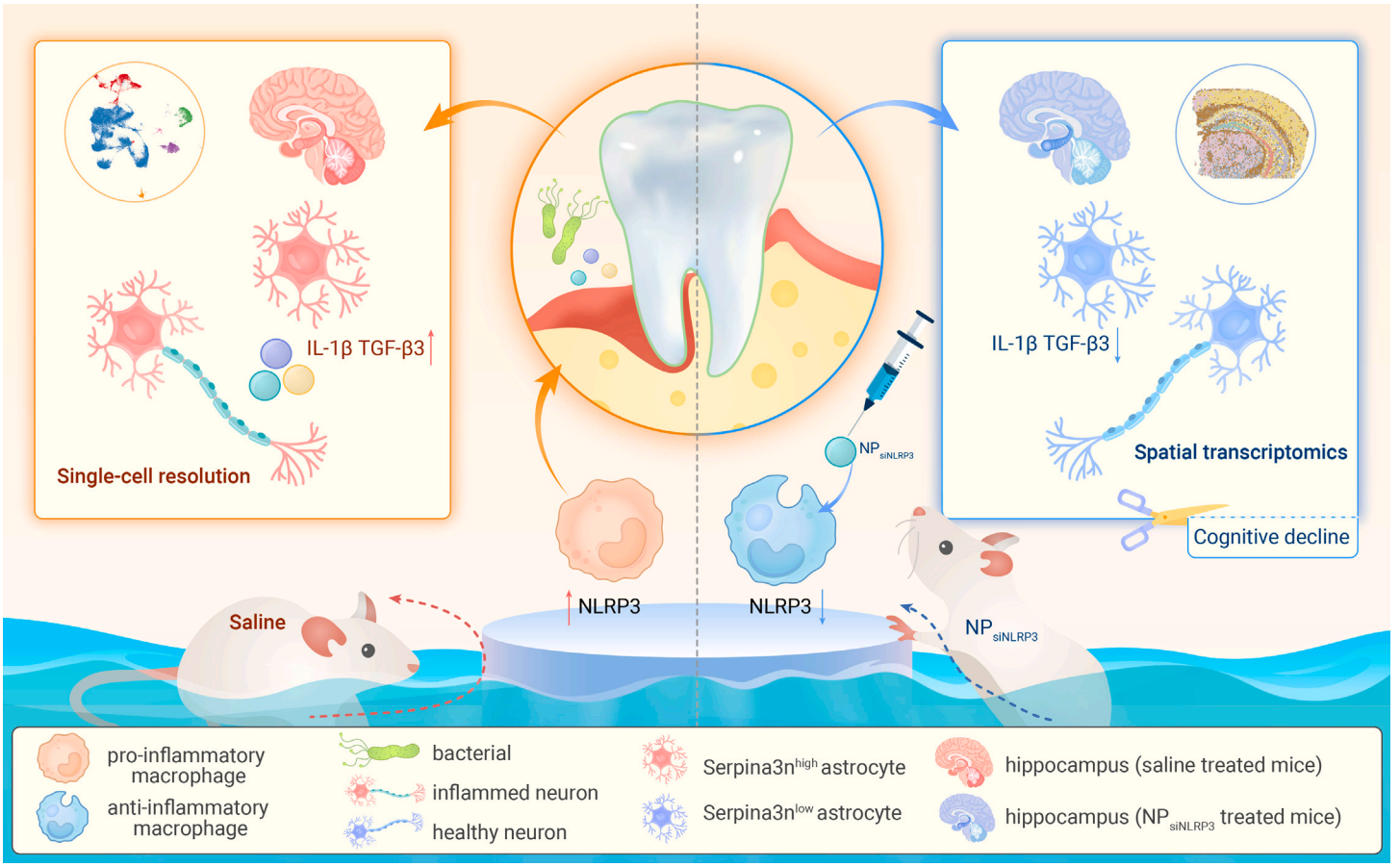
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Periodontal tissue damage is linked to cognitive decline.
- Impaired macrophages disrupt periodontal tissue homeostasis.
- Inhibition of the NLRP3 inflammasome restores periodontal macrophage function and prevents cognitive decline.
- Nanoparticle-mediated restoration of gingival macrophage function is a novel treatment for periodontitis-related cognitive decline.

REPORT

Restoring periodontal tissue homoeostasis prevents cognitive decline by reducing the number of Serpina3n^{high} astrocytes in the hippocampus

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Cognitive decline has been linked to periodontitis through an undetermined pathophysiological mechanism. This study aimed to explore the mechanism underlying periodontitis-related cognitive decline and identify therapeutic strategies for this condition. Using single-nucleus RNA sequencing we found that changes in astrocyte number, gene expression, and cell–cell communication were associated with cognitive decline in mice with periodontitis. In addition, activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome was observed to decrease the phagocytic capability of macrophages and reprogram macrophages to a more proinflammatory state in the gingiva, thus aggravating periodontitis. To further investigate this finding, lipid-based nanoparticles carrying NLRP3 siRNA ($NP_{siNLRP3}$) were used to inhibit overactivation of the NLRP3 inflammasome in gingival macrophages, restoring the oral microbiome and reducing periodontal inflammation. Furthermore, gingival injection of $NP_{siNLRP3}$ reduced the number of Serpina3n^{high} astrocytes in the hippocampus and prevented cognitive decline. This study provides a functional basis for the mechanism by which the destruction of periodontal tissues can worsen cognitive decline and identifies nanoparticle-mediated restoration of gingival macrophage function as a novel treatment for periodontitis-related cognitive decline.

INTRODUCTION

Periodontitis is a widespread chronic inflammatory condition that can lead to the disruption of periodontal tissue homoeostasis, tooth loss, and exacerbation of extraoral diseases such as colitis[1] and cardiovascular diseases.[2] Increasing evidence has suggested the involvement of periodontitis in the pathogenesis of cognitive decline.[3,4] Compared with people without periodontitis, patients with periodontitis have been reported to exhibit significant cognitive decline.[5] It is thought that disruption of periodontal tissue homoeostasis leads to the entry of periodontal pathogens and related toxic substances into the brain, causing neuroinflammation and resulting in cognitive decline.[4,6] However, further research is needed to understand the exact changes in brain physiology and the underlying mechanisms.

Recent research has highlighted the important role of astrocytes in the development of cognitive decline.[7] The hippocampus is a region of the brain that plays an integral role in spatial learning and memory, and hippocampal dysfunction has been linked to cognitive deficits and increased susceptibility to Alzheimer disease (AD).[8] Astrocytes are abundant in the hippocampus and provide nutrients to neurons and maintain homoeostasis.[9] However, when astrocytes become reactive in response to disease, they can cause cognitive decline.[9] For instance, neuroinflammation can activate A1 astrocytes, which express high levels of genes that result in damage to synapses and impair cognitive function.[9,10] However, involvement of reactive astrocytes in the pathogenesis of periodontitis-related cognitive decline remains uncharacterized. To further investigate the various mechanisms by which these brain cells interact and disrupt homoeostasis, leading to cognitive decline, single-nucleus RNA sequencing (snRNA-seq) and spatial transcriptome RNA-seq (stRNA-seq) were used to construct a comprehensive spatial cell atlas of the brain. This approach is anticipated to provide insight into the mechanisms of cognitive decline and its connection to periodontitis.

Macrophages are essential for preserving periodontal tissue homoeostasis and preventing periodontitis.[11] Constituting a key class of immune cells, macrophages play pivotal roles in defending against pathogens and regulating inflammatory and immune responses in the gingiva, the protective layer encircling the teeth.[12,13] Macrophages play pivotal roles in immune surveillance and protection against pathogen invasion by expressing pattern recognition receptors, nuclear hormone receptors, and cytokine receptors.[14] However, impairment of the immunoprotective and inflammation-related/immunoregulatory functions of macrophages are closely linked to the emergence and progression of multiple inflammatory and age-related illnesses.[15,16] For instance, phenotypic modulation of macrophages increases the release of proinflammatory factors, resulting in decreased numbers of enteric neurons and enteric neural stem cells and leading to gastrointestinal dysfunction.[17] Therefore, aberrant activity of periodontal macrophages may be a major factor in the emergence and progression of periodontitis. Given that periodontitis is generally unresponsive to current clinical treatments,[18] which include mechanical debridement in combination with antibiotics,[19] it is imperative to investigate whether macrophages are effective and safe targets for treating periodontitis and mitigating its effects on cognition.

In this study, we aimed to explore the mechanism underlying periodontitis-induced cognitive decline and to identify potential therapeutic strategies for this condition. We observed that astrocytes in mice with periodontal tissue destruction exhibited significant changes in number, gene expression, and intercellular communication, which were associated with cognitive decline. In addition, we discovered that NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation in gingival macrophages impairs the capacities of these cells for host defense and inflammation control, thus exacerbating periodontal tissue destruction. Moreover, we found that inhibiting the NLRP3 inflammasome in gingival macrophages restored periodontal tissue homoeostasis and prevented cognitive decline by reducing the number of Serpina3n^{high} astrocytes in the hippocampus. Our findings not only elucidate the role of macrophage dysfunction in the disruption of periodontal tissue homoeostasis but also provide a basis for the development of an effective therapeutic approach to ameliorate periodontitis and mitigate its negative effect on cognition.

RESULTS

Disruption of periodontal tissue homoeostasis causes cognitive impairment and an increase in the astrocyte number in the hippocampus

We established a ligature-induced periodontitis model in 12-month-old mice to explore the potential correlation between periodontitis and cognitive decline (Figure 1A). At 6 weeks after oral ligature placement, there was a marked decrease in the amount of alveolar bone, a key indicator of periodontal tissue disruption (Figures 1B and 1C). To evaluate the cognitive function of the mice, we measured their escape latency in the Morris water maze (MWM) test. Compared with healthy mice, mice with periodontitis had an obvious increase in escape latency (Figures 1D and 1E), suggesting that periodontitis may exacerbate cognitive decline in mice.

Research has shown that the destruction of periodontal tissue can lead to hippocampal dysfunction, which can cause cognitive difficulties and an

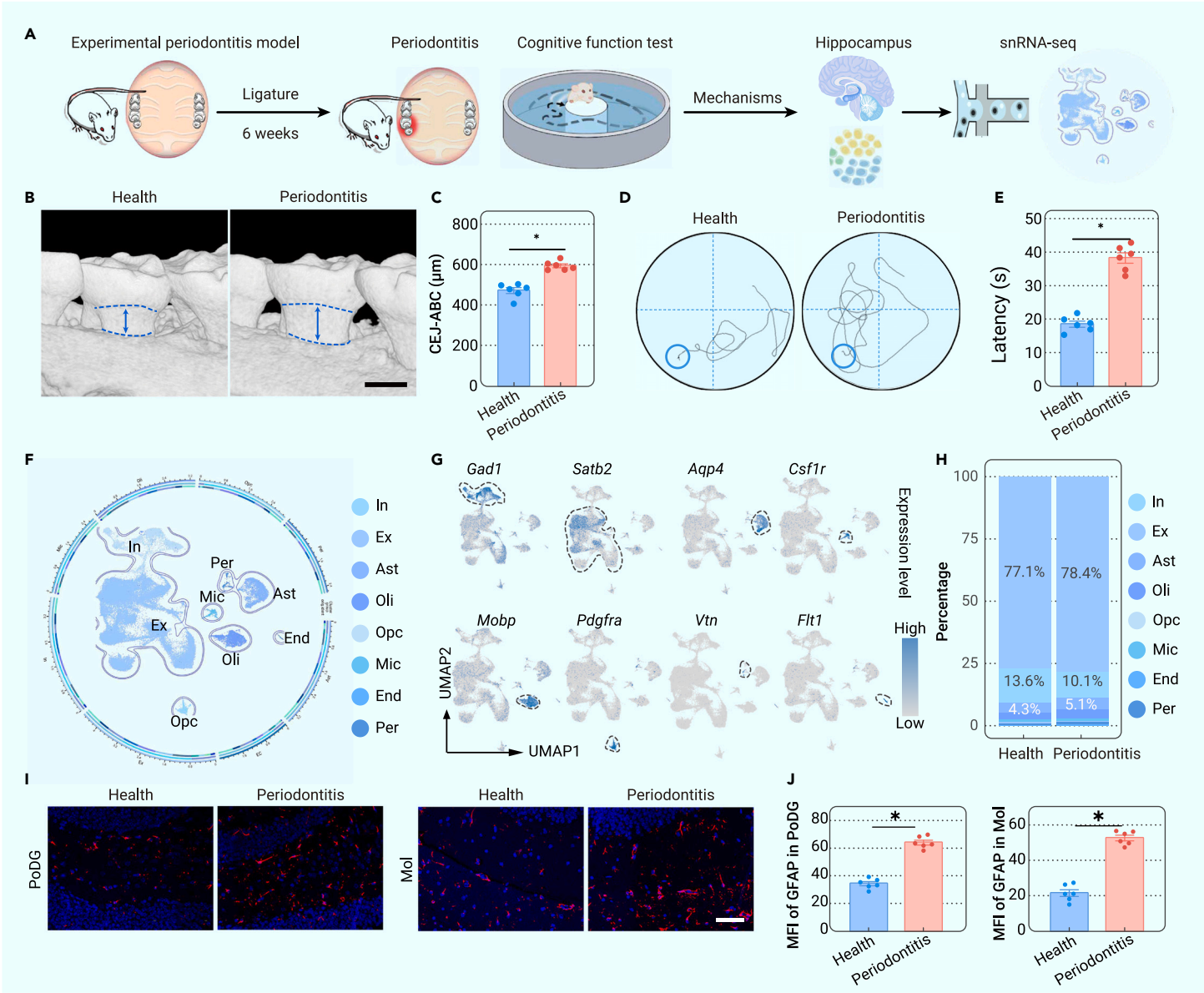

Figure 1. Disruption of periodontal tissue homoeostasis causes cognitive impairment and an increase in the astrocyte number in the hippocampus (A) Overview of the experimental design. (B and C) Three-dimensional (3D) reconstructions of the mandible in each group generated by microcomputed tomography (μCT). The vertical line extends from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC). The CEJ-ABC distance was measured on both the buccal and palatal sides. Scale bar, 500 μm. Quantification of the CEJ-ABC distance in interradicular alveolar bone in the healthy and periodontitis groups. (D and E) Representative traces of and latency data for mice in each group in the probe phase of the MWM test. (F) Circle plot showing the distribution of different cell types in the brains of mice in each group. The cell clusters are color coded and were annotated post hoc based on their transcriptional profiles. (G) UMAP plots showing the expression profiles of the indicated specific marker genes for the corresponding cell types. (H) Histogram showing the proportions of the corresponding cell types. (I and J) GFAP$^+$ cells in the PoDG and Mol subregions of the hippocampus of mice in each group were detected by IF staining and quantified. Nuclei were stained with DAPI. Scale bar, 50 μm. The data are shown as the means ± SEMs of 6 samples (C, E, and J) per group. Three biological replicates were combined to establish 1 sample, and sequencing data from 2 samples were analyzed (F–H). Student's t test; *p < 0.05.

increased likelihood of developing AD.[4,6] However, the cellular and molecular changes in the hippocampus in mice with periodontal tissue destruction are unclarified. To address this gap in knowledge, we conducted snRNA-seq to determine the alterations in the cell types present in the hippocampus. Our research revealed that the hippocampus contains eight major cell types: excitatory neurons (Ex) (*Stab2*$^+$), inhibitory neurons (In) (*GAD1*$^+$), astrocytes (Ast) (*Aqp4*$^+$), microglia (Mic) (*Csf1r*$^+$), oligodendrocytes (Oli) (*Mobp*$^+$), endothelial cells (End) (*Flt1*$^+$), oligodendrocyte progenitor cells (Opc) (*Pdgfra*$^+$), and pericytes (Per) (*Vtn*$^+$) (Figures 1F, 1G, and S1). Our study revealed that the proportion of astrocytes, one of the three most prevalent cell types, was increased in the hippocampus in mice with periodontitis (Figure 1H). Furthermore, immunofluorescence (IF) staining revealed that the number of astrocytes was significantly higher in multiple subregions of the hippocampus in these mice (Figures 1I and 1J). Collectively, our data imply that cognitive impairment may be associated with an increased number of astrocytes in the hippocampus in mice with periodontal tissue destruction.

Astrocytes in mice with periodontal tissue damage exhibit alterations that are linked to cognitive decline

The above results show that periodontal tissue damage can lead to cognitive decline, which is associated with an increase in the number of astrocytes in the hippocampus. We then focused on the functional changes in astrocytes in the hippocampus following periodontitis induction. Notably, the terms axonogenesis, axon guidance, cell junction assembly, learning, and associative learning were enriched in the upregulated differentially expressed genes (DEGs) in astrocytes in the healthy group (Figure 2A). Conversely, the terms Alzheimer disease, ferroptosis, and cellular response to extracellular stimuli, which have been associated with cognitive decline, were enriched in the upregulated DEGs in astrocytes in the periodontitis group (Figure 2B). Analysis of the scores of each pathway gene revealed that the expression of genes associated with learning was reduced but the expression of genes associated with AD, ferroptosis, and the cellular response to extracellular stimuli was increased in astrocytes in the periodontitis group compared to the healthy group (Figure 2C).

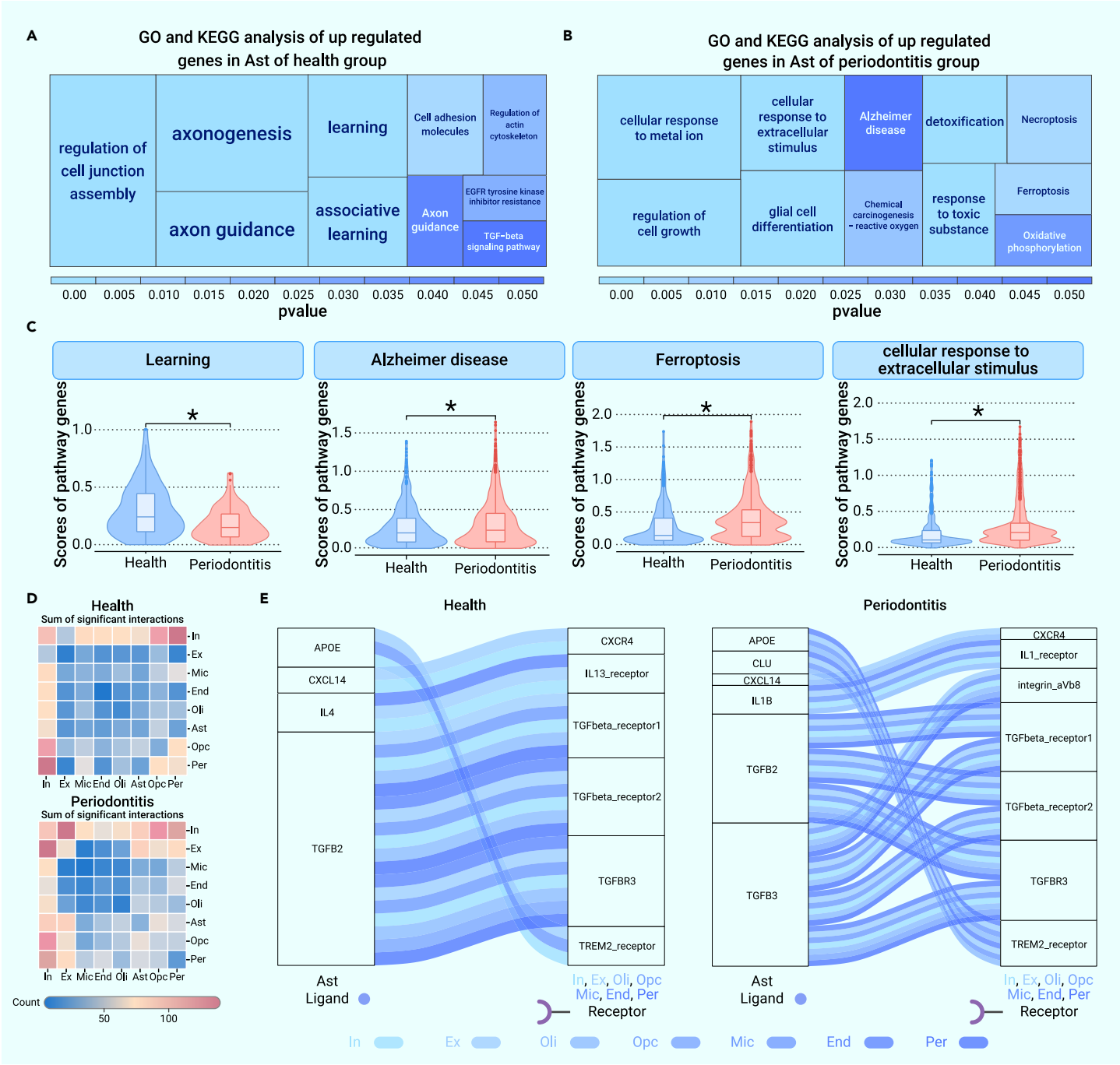

Figure 2. Astrocytes in mice with periodontal tissue damage exhibit alterations that are linked to cognitive decline (A and B) GO enrichment analysis of DEGs in the hippocampus of the healthy group compared with the periodontitis group, with the dot sizes representing the number of DEGs in the enriched terms and the color scale indicating the significance of the enrichment. (C) Statistical evaluation of the scores of genes involved in learning, AD, ferroptosis, and the cellular response to external stimuli. (D) Heatmap showing the numbers of ligand–receptor pairs between the main cell types in the mouse hippocampus. (E) River plot showing the chosen ligand–receptor pairs in the major cell types in the mouse hippocampus. Three biological replicates were combined to establish 1 sample, and sequencing data from 2 samples were analyzed (A–E). Student's t test; *p < 0.05.

Abnormalities in intercellular communication in the hippocampus have been observed to be linked to a decrease in cognitive function.[7,20] Therefore, we used CellPhoneDB to investigate intercellular interactions in the hippocampus. We identified strong interactions between astrocytes and cells in other subclusters, particularly the inhibitory neuron and excitatory neuron clusters (Figures 2D and S2). Moreover, we identified ligand–receptor pairs between astrocytes and other cell types. In periodontitis model mice, astrocytes had the greatest number of potential interactions with In, Ex, End, and Mic, which are involved in cognitive decline–related signaling pathways mediated through interactions such as the *CLU-TREM2*, *IL1B-IL1R*, *TGFB3-Integrin αVβ8*, *TGFB3-TGFBR1*, *TGFB3-TGFBR2*, and *TGFB3-TGFBR3* interactions (Figure 2E). Our findings suggest that astrocytes, which may be involved in cognitive decline resulting from periodontal tissue damage, exhibit an increased activity of pathways associated with cognitive decline and abnormal signaling.

Activation of NLRP3 signaling exacerbates periodontal tissue damage due to impairment of macrophage function

We conducted RNA-seq analysis of the periodontal tissues of mice in the healthy and periodontitis groups to investigate the mechanism underlying the increased number of astrocytes in the hippocampus and the aggravation of cognitive decline caused by periodontitis. Gene Ontology (GO) enrichment analysis revealed that the upregulated DEGs in the gingiva of mice in the healthy group were associated with cell growth, fibroblast proliferation, and stem cell population maintenance (Figure 3A). In contrast, the upregulated DEGs in the gingiva of mice in the periodontitis group were linked to the pattern recognition

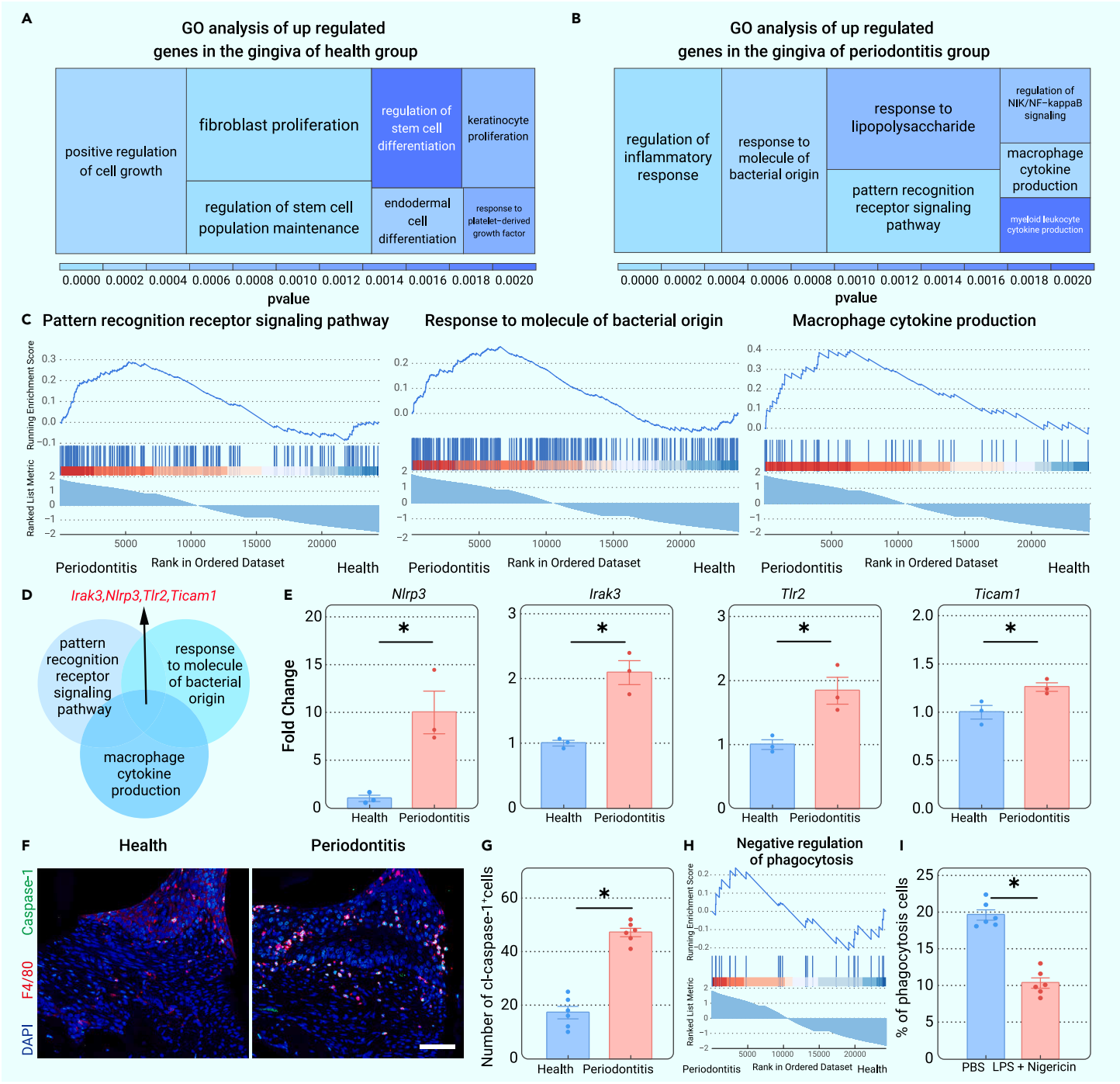

Figure 3. Activation of NLRP3 signalling exacerbates periodontal tissue damage due to impairment of macrophage function (A and B) GO enrichment analysis of DEGs in the gingiva in the healthy group compared to the periodontitis group; the number of DEGs in the enriched terms is indicated by the dot size. The color scale indicates the significance of the enrichment of each term. (C) GSEA of upregulated genes associated with pattern recognition receptor signaling and cytokine secretion. (D) Identification of genes associated with the terms pattern recognition receptor signaling pathway, response to molecules of bacterial origin, and macrophage cytokine production. (E) Histogram showing the expression of *Nlrp3, Irak3, Tlr2,* and *Ticam1* in each group. (F and G) Representative IF images and counts of F4/80$^+$ and cl-caspase-1$^+$ cells in the gingiva in each group. Nuclei were stained with DAPI. Scale bar, 50 μm. (H) GSEA comparing the activity of the selected pathways between the periodontitis group and the healthy group. (I) Flow cytometry plots of BMDMs that engulfed beads, along with the percentage of phagocytic cells in each group. The data are representative of 3 biological replicates (A–E) or are displayed as the means ± SEMs of 6 samples (G and I) per group. Student's t test; *p < 0.05.

receptor signaling pathway, the response to molecules of bacterial origin and macrophage cytokine production (Figure 3B). Gene set enrichment analysis (GSEA) was conducted to identify biological processes associated with genes upregulated in periodontitis, including the pattern recognition receptor signaling pathway, the response to molecules of bacterial origin, and macrophage cytokine production (Figure 3C).

Subsequent analysis of the RNA-seq data revealed that the expression of *Nlrp3, Irak3, Tlr2,* and *Ticam1* was significantly increased, by 10.1-, 2.09-, 1.84-, and 1.27-fold, respectively, in the periodontitis group (Figures 3D and 3E). In addition, IF staining showed that caspase-1, a marker of NLRP3 inflammasome activation, was highly expressed in gingival macrophages and that its expression was significantly increased in the gingiva of periodontitis model mice (Figures 3F and 3G). qRT-PCR analysis further confirmed that the *Nlrp3* expression level was significantly higher in the periodontal tissues of periodontitis model mice than in those of mice without periodontitis (Figure S3). Flow cytometric analysis demonstrated that the secretion of interleukin (IL)-1β, an indicator of NLRP3 inflammasome activation, by macrophages in the gingiva of periodontitis model

mice was greatly increased after *ex vivo* stimulation with lipopolysaccharides (LPS) (Figure S4).

Research has demonstrated that a decrease in the phagocytic capacity of macrophages is associated with the exacerbation of periodontitis and subsequent damage to periodontal tissue.[21] GSEA revealed that the negative regulation of the phagocytosis gene set was enriched in the periodontitis group compared to the healthy group (Figure 3H). This finding prompted us to investigate whether NLRP3 inflammasome activation could be responsible for the decreased phagocytic capacity of macrophages. We found that bone marrow–derived macrophages (BMDMs) stimulated with NLRP3 activators had reduced phagocytic capacity (Figures 3I and S5A–S5C). Collectively, these results suggest that NLRP3 activation decreases the phagocytic capacity of macrophages and increases the secretion of inflammatory cytokines by macrophages, thus exacerbating periodontitis.

Injecting $NP_{siNLRP3}$ into the gingiva prevents periodontal tissue damage and associated cognitive decline

We previously demonstrated that lipid-based PEGb-PLA nanoparticles ($NP_{siNLRP3}$) can be enabled to target macrophages by manipulating the surface charge and PEG density.[22] To deliver siNLRP3 into macrophages while preventing its degradation, we synthesized $NP_{siNLRP3}$) (Figure 4A). Transmission electron microscopy (TEM) revealed that the $NP_{siNLRP3}$ were spherical, with a diameter of 122.3 ± 3.2 nm (Figures 4B and 4C). In addition, the zeta potential and the size of $NP_{siNLRP3}$ remained stable over a 48-h period (Figure S6). Furthermore, siNLRP3 was successfully encapsulated in the NPs, with an encapsulation efficiency of greater than 90% (Table S1). These analyses were performed in preparation for investigating whether NLRP3 inflammasome activation leads to macrophage dysfunction and worsens periodontitis and to assess whether reducing *Nlrp3* expression in gingival macrophages can mitigate these effects.

We assessed the impact of suppressing NLRP3 inflammasome activation with $NP_{siNLRP3}$. qRT-PCR and western blot analyses demonstrated that $NP_{siNLRP3}$ inhibited NLRP3 expression in BMDMs exposed to the NLRP3 activators LPS and nigericin (Figures 4D and S7). Moreover, western blot analysis showed that the levels of the IL-1β precursor (pro-IL-1β) and cleaved caspase-1 (cl-caspase-1) were decreased in the supernatant of $NP_{siNLRP3}$-treated BMDMs compared to the supernatant of NP_{siNC}-, $Free_{siNLRP3}$- or PBS-treated BMDMs (Figures 4D; Table S2). In addition, qRT-PCR analysis showed that *Nlrp3* mRNA expression in gingival macrophages was significantly lower in $NP_{siNLRP3}$-treated mice than in saline- or free siNLRP3-treated mice (Figure S8; Table S3). Furthermore, flow cytometric analysis showed that treatment with $NP_{siNLRP3}$ decreased IL-1β secretion from gingival macrophages (Figures S9A and S9B). Taken together, these results suggest that $NP_{siNLRP3}$ effectively disrupts NLRP3 activation in macrophages both *in vitro* and *in vivo*.

Subsequently, we assessed the impact of $NP_{siNLRP3}$ on the phagocytic capacity of macrophages. As anticipated, treatment with $NP_{siNLRP3}$ increased the phagocytic ability of macrophages (Figures 4E and 4F). Because macrophage dysfunction is associated with oral dysbiosis and persistent inflammation in the gingiva,[23] we examined the effects of $NP_{siNLRP3}$ on the oral microbiome via 16S rRNA-seq. Our findings demonstrated that the alpha diversity of the oral microbiome was significantly reduced in $NP_{siNLRP3}$-treated periodontitis model mice compared to saline-treated periodontitis model mice (Figure S10). At the phylum level, treatment with $NP_{siNLRP3}$ decreased the abundance of *Bacteroidota*, which includes various periodontitis-related bacteria, such as *Porphyromonas gingivalis* and *Treponema denticola*. Moreover, LEfSe (Linear Discriminant Analysis Effect Size) revealed that Bacteroidota species were dominant in saline-treated periodontitis model mice, and Proteobacteria species were dominant in $NP_{siNLRP3}$-treated periodontitis model mice (Fig

ure 4G).

Furthermore, injection of $NP_{siNLRP3}$ into the gingiva successfully reduced inflammation and alveolar bone loss in mice with periodontitis (Figures 4H and 4I). In addition, the number of $TRAP^+$ osteoclasts in the periodontium of $NP_{siNLRP3}$-treated periodontitis model mice was decreased (Figure S11). Moreover, the cognitive function of these mice was improved, as indicated by the decrease in the escape latency in the MWM test (Figures 4J and 4K). Finally, biosafety evaluation showed that the major organs of $NP_{siNLRP3}$-treated mice were healthy (Figure S12). These results suggest that $NP_{siNLRP3}$ can effectively and safely prevent periodontitis in mice by increasing the phagocytic capacity of macrophages and thus preventing the associated cognitive decline.

The number of $Serpina3n^{high}$ astrocytes in the hippocampus of periodontitis model mice is significantly reduced after periodontal treatment with $NP_{siNLRP3}$

The present study revealed that changes in astrocyte number and gene expression are associated with periodontal tissue damage in mice. To assess the effects of treatment with $NP_{siNLRP3}$ on the number of specific astrocyte subsets in the hippocampus and the gene expression patterns in these cells, we divided astrocytes into four subsets (Ast-1, Ast-2, Ast-3, and Ast-4) (Figures 5A and 5B). Each subset was identified by specific markers (Figure 5C). Notably, the proportion of the Ast-1 subset was significantly reduced after treatment with NPsiNLRP3 (Figure 5D). To determine the functions of each subset of astrocytes, we compared them to previously defined astrocyte subtypes, such as inflammatory/A1 astrocytes and AD-related astrocytes. We found that the Ast-1 subset exhibited significantly higher expression of inflammation/A1-related genes and AD-related genes than the other astrocyte subsets (Figure 5E). In addition, the expression of the markers of reactive astrocytes *Gfap* and *Serpina3n* was significantly higher in the Ast-1 subset than in the other astrocyte subsets (Figure 5F). Because *Serpina3n* was specifically expressed in the Ast-1 subset, we referred to the cells in this subset as Serpina3nhigh astrocytes. *Serpina3n* encodes anti-chymotrypsin, which is associated with amyloid accumulation in AD.[24] Thus, Serpina3nhigh astrocytes may be linked to cognitive decline.

To investigate the developmental state of Serpina3nhigh astrocytes, we used the R package monocle3 to construct their developmental trajectory. The pseudotime trajectory revealed that Serpina3nhigh astrocytes were located at the end of the trajectory, indicating that they are terminally differentiated astrocytes (Figure 5G). Furthermore, the expression of *Serpina3n* was upregulated, suggesting that Serpina3nhigh astrocytes could be differentiated from cells of the Ast-2 subset (Serpina3nlow astrocytes) (Figure 5H).

Gene set variation analysis (GSVA) revealed that the gene set—positive regulation of tau protein kinase activity was enriched in Serpina3nhigh astrocytes, indicating a potential association of these cells with cognitive decline (Figure 5I). Conversely, dopamine neurotransmitter receptor activity was enriched in Serpina3nlow astrocytes, suggesting that they represent a homoeostatic population of astrocytes (Figure 5I). The uniform manifold approximation and projection (UMAP) plot further confirmed that the number of cognitive decline–associated Serpina3nhigh astrocytes was reduced after periodontal treatment with NPsiNLRP3 (Figure S13A). In addition, the violin plot showed that the expression of *Serpina3n* was significantly reduced in NPsiNLRP3-treated mice compared with saline-treated mice (Figure S13B). Collectively, these results suggest that periodontal treatment with NPsiNLRP3 may improve cognitive function by reducing the proportion of Serpina3nhigh astrocytes.

Overexpression of Serpina3n in astrocytes abolishes the beneficial effects of periodontitis treatment on cognitive decline in mice

We conducted spatial transcriptome RNA-seq (stRNA-seq) to visualize $Serpina3n^{high}$ astrocytes in the hippocampus and investigate their roles in cognitive decline (Figure 6A). By analysing the gene expression profiles and localization of cells in hippocampal sections, we identified the major cell types by the expression of specific markers (Figures 6B, S14, and S15) and selected astrocyte spots for further study. We then assigned each astrocyte spot a $Serpina3n^{high}$ astrocyte signature score based on stRNA-seq and snRNA-seq data (Figures 6C and 6D). In these figures, cells with high signature scores are indicated in red, while those with low signature scores are indicated in blue. We observed that astrocyte spots with high $Serpina3n^{high}$ astrocyte signature scores were distributed throughout the hippocampus and that the number of these spots was reduced after periodontal treatment with $NP_{siNLRP3}$ (Figure 6D). The ridgeline plot further showed that the $Serpina3n^{high}$ astrocyte signature scores were lower in $NP_{siNLRP3}$-treated mice than in saline-treated mice (Figure 6E). The number of spots exhibiting a high expression of *Serpina3n* was also reduced in $NP_{siNLRP3}$-treated mice compared to saline-treated mice (Figure 6F), and the expression of *Serpina3n* in astrocytes was significantly decreased after treatment with $NP_{siNLRP3}$ (Figures 6G and 6H). IF staining further confirmed that periodontal treatment with $NP_{siNLRP3}$ significantly decreased the number of $Serpina3n^{high}$ astrocytes in multiple subregions of the hippocampus (Figure S16).

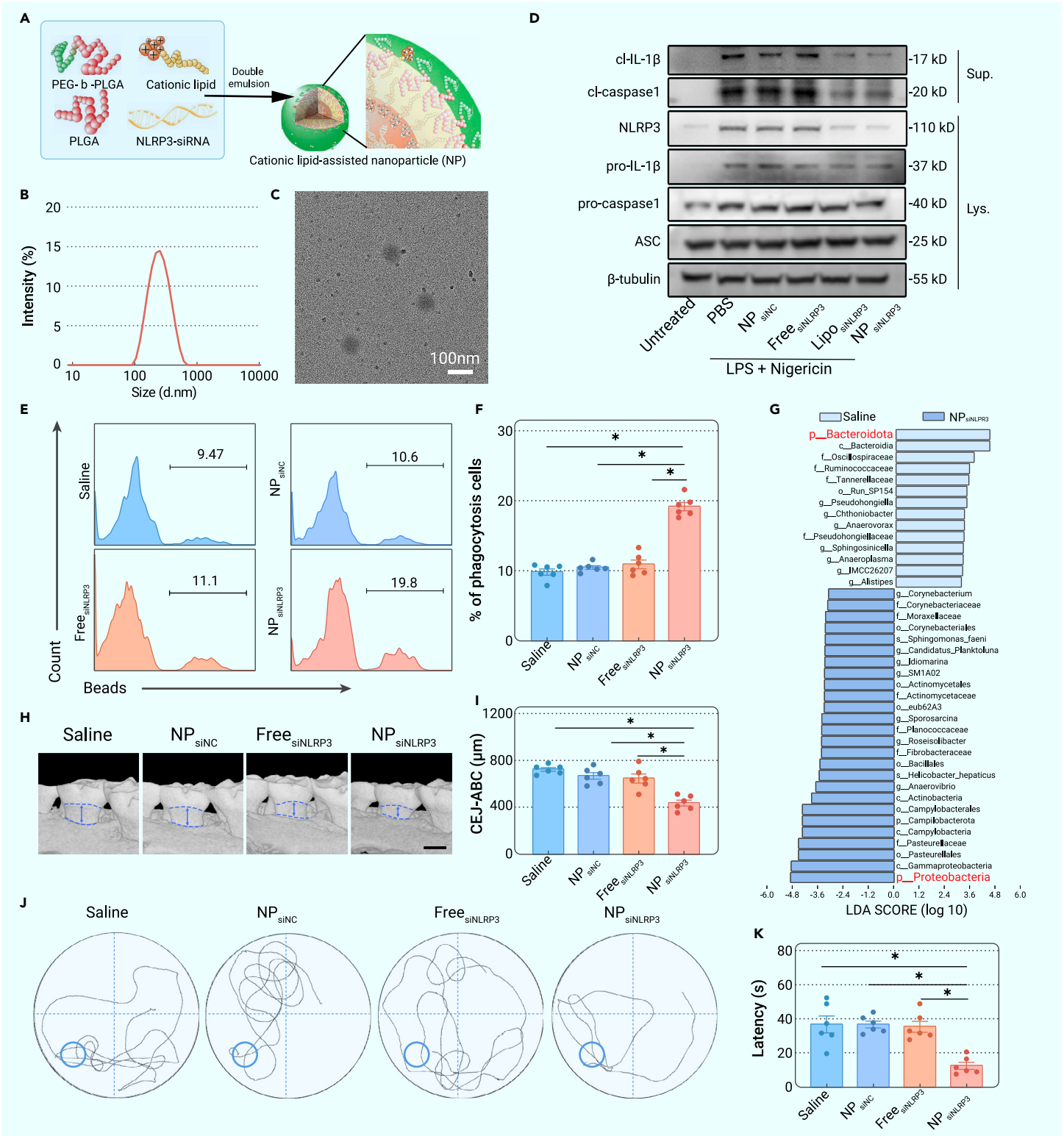

Figure 4. Injecting $NP_{siNLRP3}$ into the gingiva prevents periodontal tissue damage and associated cognitive decline (A) $NP_{siNLRP3}$ were prepared with siNLRP3, BHEM-Chol, PEG5K-b-PLGA11K, or PLGA11K by the double emulsion method. (B) The diameter and distribution of $NP_{siNLRP3}$ were analyzed with the Zetasizer Nano ZS90 system. (C) The morphology of $NP_{siNLRP3}$ was analyzed by TEM. (D) Representative western blots showing the protein levels of caspase-1, cl-caspase-1, NLRP3, IL-1β, pro-IL-1β, and ASC in BMDMs treated with LPS, nigericin, and siNLRP3 or siNC. (E and F) Representative flow cytometry plots of $NP_{siRNA\text{-}cy5}$ engulfed by gingival macrophages and the percentages of phagocytic cells in saline-, NP_{siNC}-, $Free_{siNLRP3}$-, and $NP_{siNLRP3}$-treated 12-month-old mice. (G) LEfSe results showing bacterial abundances in the oral microbiome in each group. (H) 3D reconstructions of the mandible in each group generated by μCT. The vertical line extends from the CEJ to the ABC. The CEJ-ABC distance was measured on both the buccal and palatal sides. Scale bar, 500 μm. (I) Quantification of the CEJ-ABC distance in interradicular alveolar bone in each group. (J and K) Representative traces of and latency data for periodontitis model mice treated with saline, NP_{siNC}, $Free_{siNLRP3}$, and $NP_{siNLRP3}$ in the probe phase of the MWM test. The data are from 3 independent experiments (B–D) or 6 biological replicates (G) and are shown as the means ± SEMs of 6 samples (F, I, and K) per group. Two-way ANOVA; *p < 0.05.

We then investigated whether the beneficial effects of periodontal treatment with $NP_{siNLRP3}$ on cognitive decline are abolished when Serpina3n is overexpressed in astrocytes. To introduce transgenes into hippocampal astrocytes in mice, we used an adeno-associated virus (AAV) expressing the promoter of *Gfap*, which is specifically expressed in astrocytes. We used $AAV_{Serpina3n}$ and AAV_{NC} as positive and negative controls, respectively. The Serpina3n level in

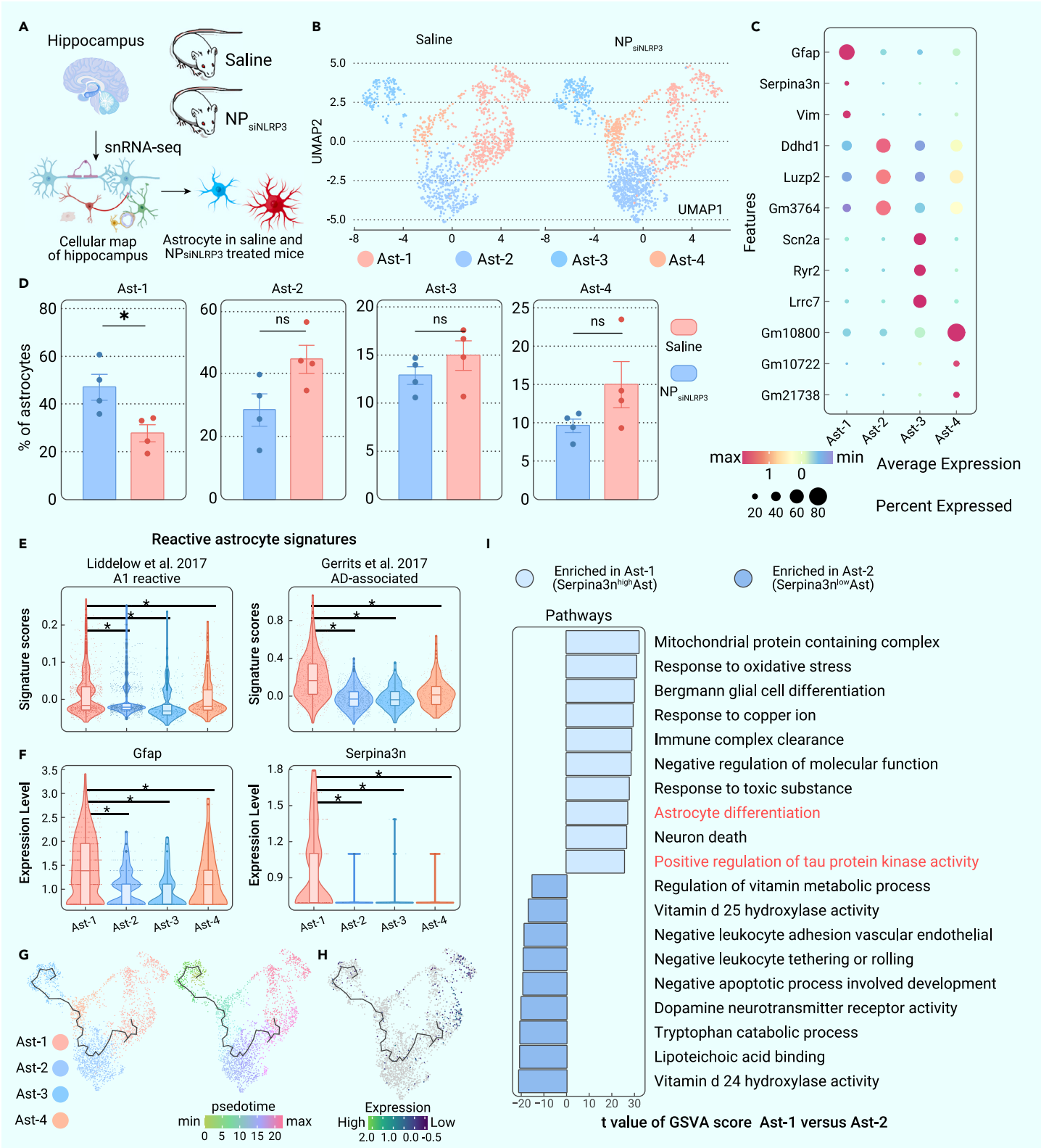

Figure 5. The number of Serpina3n^{high} astrocytes was significantly reduced in the hippocampus of periodontitis model mice after periodontal treatment with NP$_{siNLRP3}$ (A) Overview of the experimental strategy. (B) UMAP plot showing the distributions of different astrocyte types in the hippocampus of periodontitis model mice treated with saline or NP$_{siNLRP3}$. The cell clusters are color coded and were annotated post hoc based on their transcriptional profiles. (C) Dot plot showing specific marker genes of the Ast-1, Ast-2, Ast-3, and Ast-4 subsets in the hippocampus of periodontitis model mice treated with saline or NP$_{siNLRP3}$. The dot color indicates the average expression level of genes. The dot size indicates the percentage of genes expressed. (D) Histogram showing the proportions of the Ast-1, Ast-2, Ast-3, and Ast-4 subsets relative to the total astrocyte count in the hippocampus of each group. (E) Signatures of reactive astrocytes in the hippocampus of periodontitis model mice treated with saline or NP$_{siNLRP3}$. Violin plots showing the distribution of expression scores for previously defined signature genes for inflammatory/A1 astrocytes and AD-related astrocytes across the clusters. (F) Violin plots showing the distribution of *Gfap* and *Serpina3n* expression across the clusters. (G) Continuous trajectory of the Ast-1, Ast-2, Ast-3, and Ast-4 subsets in the hippocampus of periodontitis model mice treated with saline or NP$_{siNLRP3}$, with pseudotime, all subsets and individual subsets shown along the trajectory. (H) Continuous trajectory across astrocytes in the hippocampus in combination with the expression profiles of *Serpina3n*. (I) Bar plots showing differences in the activity of pathways associated with the DEGs between the Ast-1 and Ast-2 subsets in the hippocampus of periodontitis model mice treated with saline or NP$_{siNLRP3}$, as determined by GSVA. The data are from 4 biological replicates (B, C, and E–I) and are shown as the means ± SEMs (D). Student's t test or the Wilcoxon test; *p < 0.05.

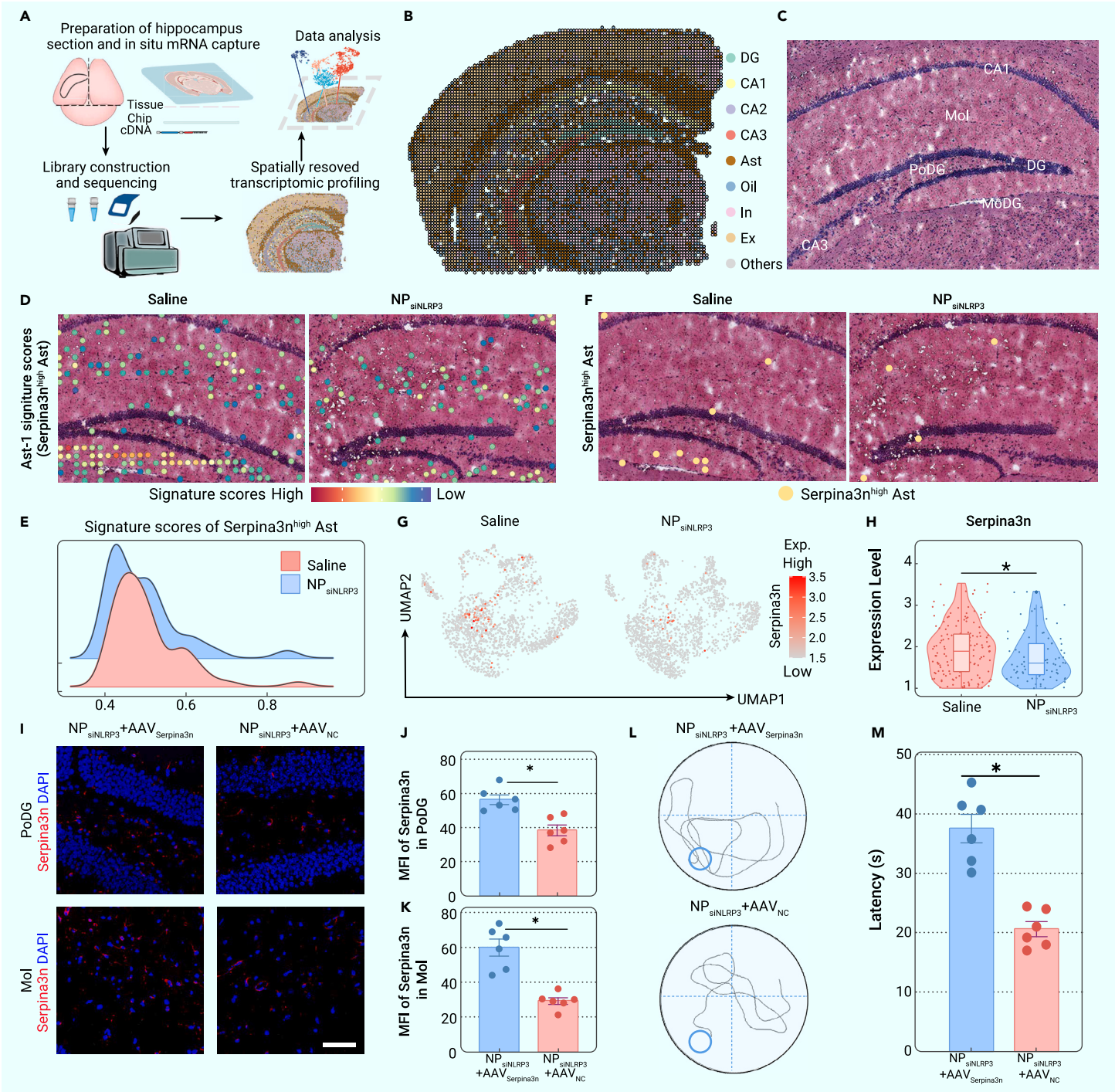

Figure 6. Overexpression of Serpina3n in astrocytes abolishes the beneficial effects of periodontal treatment on cognitive decline in mice (A) Overview of the experimental strategy. (B) Representative spatial map of the cell types in the hippocampus of periodontitis model mice treated with saline or $NP_{siNLRP3}$. (C) H&E staining showing different subregions of the mouse hippocampus. (D and E) The distribution of $Serpina3n^{high}$ astrocytes in the hippocampus was scored. Ridgeline plot showing the $Serpina3n^{high}$ astrocyte signature scores in each group. (F) Image of H&E staining in combination with a spatial map showing spots of $Serpina3n^{high}$ astrocytes in the hippocampus of periodontitis model mice treated with saline or $NP_{siNLRP3}$. (G) UMAP plot showing the expression profiles of $Serpina3n^{high}$ astrocytes in the hippocampus of periodontitis model mice treated with saline or $NP_{siNLRP3}$. (H) Statistical analysis of the expression of Serpina3n in hippocampal astrocytes in periodontitis model mice treated with saline or $NP_{siNLRP3}$. (I–K) $Serpina3n^{high}$ cells in the PoDG and Mol subregions of the hippocampus in $NP_{siNLRP3}$-treated periodontitis model mice treated with $AAV_{Serpina3n}$ or AAV_{NC} were detected by IF staining and quantified. Nuclei were stained with DAPI. Scale bar, 50 μm. (L and M) Representative traces of and latency data for $NP_{siNLRP3}$-treated periodontitis model mice treated with $AAV_{Serpina3n}$ or AAV_{NC} in the probe phase of the MWM test. The data are shown as the means ± SEMs of 6 samples (J, K, and M) per group or 1 sample (B and D–H) per group. Student's t test or the Wilcoxon test; *$p < 0.05$.

astrocytes was significantly increased in $AAV_{Serpina3n}$-treated mice compared to AAV_{NC}-treated mice (Figures 6I–6K). Furthermore, the escape latency of both $AAV_{Serpina3n}$- and $NP_{siNLRP3}$-treated mice was significantly higher than that of AAV_{NC}- and $NP_{siNLRP3}$-treated mice, indicating that overexpression of Serpina3n in astrocytes is sufficient to abolish the protective effects of periodontal treatment with $NP_{siNLRP3}$ (Figures 6L and 6M). Taken together, these findings suggest that the protective effects of $NP_{siNLRP3}$ on cognitive decline in mice with periodontal tissue damage may be due to the reduced level of Serpina3n in the hippocampus.

DISCUSSION

Our findings demonstrate that NLRP3 inflammasome activation in macrophages can cause the exacerbation of periodontal tissue damage, resulting in an imbalance in the oral microbiome that can lead to cognitive decline caused

by increased numbers of Serpina3n^{high} astrocytes. We propose that periodontal injection of $NP_{siNLRP3}$ is a potential strategy to restore periodontal tissue homoeostasis and thus protect against cognitive decline.

Recent studies have demonstrated a correlation between periodontitis and cognitive decline.[6] For example, in mouse models, the severity of periodontitis is associated with the degree of cognitive decline.[25] Human periodontitis patients with a greater number of teeth have been observed to experience a faster rate of hippocampal atrophy and cognitive decline.[26] However, the exact cells in the hippocampus affected by periodontitis remain to be identified. In the present study, we constructed a comprehensive single-nucleus transcriptomic atlas of the hippocampus using snRNA-seq. To our knowledge, this is the first study to explore periodontitis-induced transcriptomic changes in hippocampal cells at the single-cell level. Analysis of snRNA-seq data from hippocampal cells of mice with periodontitis and healthy mice of the same age revealed substantial differences in the proportion, signaling pattern, and cell–cell communication of astrocytes. Notably, receptor–ligand signaling between astrocytes and neurons, such as that mediated through the *IL1B-IL1R* and *TGFB3-TGFBR* interactions, was significantly increased in the periodontitis group compared to the healthy control group. Research has demonstrated that IL-1β and transforming growth factor (TGF)β3 secreted by astrocytes can exacerbate neurodegeneration and cognitive impairment.[27,28] Our findings, combined with those of previous studies, suggest that abnormal astrocytes may be involved in the development of periodontitis-related cognitive decline.

We conducted snRNA-seq and stRNA-seq to investigate alterations in cellular organization and the spatial profiles of astrocyte gene expression in the hippocampus after restoration of periodontal tissue homoeostasis. Our results showed that the number of Serpina3n^{high} astrocytes in the hippocampus was significantly decreased, which was associated with the amelioration of cognitive decline. Previous studies have demonstrated that the upregulation of Serpina3n in the hippocampus is linked to cognitive decline. For instance, Serpina3n has been found to be embedded in amyloid plaques, which are associated with cognitive decline in humans.[29] In addition, injecting Serpina3n into the CA1 region of the mouse hippocampus was shown to induce amyloid beta aggregation, suggesting that the upregulation of Serpina3n may exacerbate tau pathology and cause cognitive decline.[30] Our study revealed that the gene expression pattern of Serpina3n^{high} astrocytes was similar to that of previously defined inflammatory astrocytes and AD-related astrocytes, and functional analysis further indicated that Serpina3n^{high} astrocytes may increase tau protein kinase activity.

It has been demonstrated that suppressing Serpina3n expression in astrocytes can have therapeutic effects on learning and memory deficits.[31] For instance, the downregulation of Serpina3n in astrocytes restored cognitive abilities in postmenopausal mice.[32] Furthermore, AAV-mediated silencing of Serpina3n in astrocytes reduced blood–brain barrier dysfunction and cognitive impairment associated with tumor necrosis factor (TNF) treatment.[31] Our study revealed that the overexpression of Serpina3n in astrocytes counteracted the beneficial effects of periodontal treatment on cognitive function, suggesting that a high level of Serpina3n in astrocytes may be a critical factor in the progression of periodontitis-induced cognitive decline. Taken together, these findings suggest that Serpina3n could be a therapeutic target for cognitive decline.

Given the difficulty of targeting hippocampal astrocytes directly, restoring periodontal homoeostasis is the safest and most efficient way to reverse astrocyte abnormalities and thus ameliorate cognitive impairment. However, approaches to safely and effectively reduce inflammation and restore microbial homoeostasis in periodontal tissue remain undefined. Although broad-spectrum antibiotics such as moxifloxacin are often used to control pathogenic bacteria, their use may lead to antibiotic resistance and oral dysbiosis.[33] Periodontal macrophages are important in defending against invading microbes and preventing systemic infections but do not cause oral dysbiosis.[34] Therefore, we focused on reversing the dysfunction of gingival macrophages to restore periodontal tissue homoeostasis. Our RNA-seq data revealed the potential central role of the NLRP3 inflammasome in causing gingival macrophage dysfunction. Activation of the NLRP3 inflammasome has been associated with an increase in IL-1β production by macrophages, which can lead to age-related alveolar bone loss and periodontal inflammation.[35] Our study showed that $NP_{siNLRP3}$ can normalize the polarization and phagocytic ability of gingival macrophages, shift the oral microbiome toward a healthier and younger state, and alleviate periodontitis in mice. Thus, our findings suggest that targeting NLRP3 may be a safe and effective means of reducing periodontal inflammation and reestablishing the oral microbial homoeostasis. Moreover, surprisingly, our research showed that cognitive impairment could be ameliorated through periodontal tissue healing and regeneration by restoring macrophage function in mice. Thus, gingival macrophages are essential for restoring the homoeostasis of periodontal tissues and preventing systemic diseases and pathological processes associated with periodontitis, such as cognitive decline.

A limitation of our study is that the connection between gingival inflammation and hippocampal inflammation was not explored. Studies have revealed that periodontitis can worsen cognitive impairment through two main pathways: the infiltration of oral microorganisms into the brain and an increase in systemic inflammation. For instance, *P. gingivalis*, a key causative organism of periodontitis, has been detected in the hippocampus of patients with AD, and its abundance is correlated with the severity of tau pathology.[4] In addition, the transport of *P. gingivalis* to the hippocampus has been associated with increased oxidative stress, which can lead to microstrokes and cognitive impairment in animal models.[36] Furthermore, periodontal pathogens have been observed to cause increases in the levels of TNF-α and IL-1β in the systemic circulation, resulting in neuroinflammation in the hippocampus and cognitive decline.[37] Further research is needed to gain a better understanding of the mechanism by which astrocytes are activated in periodontitis to establish effective therapeutic strategies for both periodontitis and cognitive decline.

In summary, our findings show that impairment of the host defense and inflammation modulation functions of gingival macrophages can be reversed by inhibiting NLRP3 inflammasome signaling. Improving macrophage function can ameliorate periodontitis and prevent cognitive decline. Consequently, the use of $NP_{siNLRP3}$ to restore macrophage function can be beneficial for the treatment or prevention of aging-related periodontitis and cognitive decline.

MATERIALS AND METHODS

Materials and methods related to this work are available in the supplemental information.

DATA AND CODE AVAILABILITY

All of the data necessary to evaluate the conclusions of the article are included in the article or in the supplemental information.

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AUTHOR CONTRIBUTIONS

B.C., X.S., and Z.L. provided funding, designed the study, and edited the manuscript and figures. Z.S. provided funding, performed the experiments, analyzed the data, and drafted the manuscript. S.K. and Y.Z. performed the majority of the experiments and analyzed the data. J.C., Y.H., and S.H. analyzed the data. S.W. prepared and characterized the materials. C.X., M.Z., J.W., and C.Z. edited the manuscript. All of the authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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