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TRANSLATIONAL SCIENCE

Microglia activation in the presence of intact blood–brain barrier and disruption of hippocampal neurogenesis via IL-6 and IL-18 mediate early diffuse neuropsychiatric lupus

Dionysis Nikolopoulos ,[1,2] Theodora Manolakou ,[1,2] Alexia Polissidis,[3] Anastasia Filia,[1] George Bertsias,[4,5] Yassemi Koutmani,[3] Dimitrios T Boumpas [1,2,6]

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For numbered affiliations see end of article.

Correspondence to
Dr Dionysis Nikolopoulos, National and Kapodistrian University of Athens, Athens, Greece;
dsnikolopoulos@gmail.com
Dr Dimitrios T Boumpas;
boumpasd@uoc.gr

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ABSTRACT

Introduction Inflammatory mediators are detected in the cerebrospinal fluid of systemic lupus erythematosus patients with central nervous system involvement (NPSLE), yet the underlying cellular and molecular mechanisms leading to neuropsychiatric disease remain elusive.

Methods We performed a comprehensive phenotyping of NZB/W-F1 lupus-prone mice including tests for depression, anxiety and cognition. Immunofluorescence, flow cytometry, RNA-sequencing, qPCR, cytokine quantification and blood–brain barrier (BBB) permeability assays were applied in hippocampal tissue obtained in both prenephritic (3-month-old) and nephritic (6-month-old) lupus mice and matched control strains. Healthy adult hippocampal neural stem cells (hiNSCs) were exposed *ex vivo* to exogenous inflammatory cytokines to assess their effects on proliferation and apoptosis.

Results At the prenephritic stage, BBB is intact yet mice exhibit hippocampus-related behavioural deficits recapitulating the human diffuse neuropsychiatric disease. This phenotype is accounted by disrupted hippocampal neurogenesis with hiNSCs exhibiting increased proliferation combined with decreased differentiation and increased apoptosis in combination with microglia activation and increased secretion of proinflammatory cytokines and chemokines. Among these cytokines, IL-6 and IL-18 directly induce apoptosis of adult hiNSCs ex vivo. During the nephritic stage, BBB becomes disrupted which facilitates immune components of peripheral blood, particularly B-cells, to penetrate into the hippocampus further augmenting inflammation with locally increased levels of IL-6, IL-12, IL-18 and IL-23. Of note, an interferon gene signature was observed only at nephritic-stage.

Conclusion An intact BBB with microglial activation disrupting the formation of new neurons within the hippocampus represent early events in NPSLE. Disturbances of the BBB and interferon signature are evident later in the course of the disease.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hippocampus is a target organ of the activated immune system in NPSLE directly linked to depression, anxiety and loss of memory.
⇒ A characteristic, conserved feature of hippocampus is its neurogenic activity, which contributes to its functional plasticity.

WHAT THIS STUDY ADDS

⇒ The NZB/W-F1 lupus strain exhibits hippocampal-linked behavioural perturbations recapitulating human NPSLE thus validating it as an animal model of NPSLE.
⇒ Disturbance of the blood–brain barrier (BBB) is not a prerequisite for NPSLE; intrinsic activation of microglia may initiate the disease at early stages in the presence of an intact BBB.
⇒ Activation of microglia results in the production of IL-6 and IL-18, which directly induce apoptosis of adult hippocampal neural stem cells and induces the hippocampal-linked behavioural perturbations.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Microglia may represent a potential therapeutic strategy in NPSLE.

INTRODUCTION

Diffuse neuropsychiatric manifestations such as cognitive dysfunction, mood disorders and anxiety, are common in patients with SLE and pose significant diagnostic and therapeutic challenges.[1] From a clinical standpoint, the involvement of inflammatory mechanisms in diffuse NPSLE is critical for the subsequent therapeutic decisions.[2]

A crucial brain region implicated in NPSLE is hippocampus, a complex structure associated with cognitive functions such as memory and regulation of mood, while its dysfunction has been linked to various neuropsychiatric disorders.[3 4] Previous studies have suggested that lupus autoantibodies induce a spectrum of pathologies in the hippocampus including aberrant excitatory signalling, neuronal apoptosis and dendritic pruning.[5–8] Neuroimaging studies have also shown hippocampal hypermetabolism in lupus, which is associated with impaired memory performance and mood alterations.[9] Hippocampal atrophy is also evident in lupus patients with longstanding-disease and cognitive dysfunction.[10] The underlying molecular mechanisms for hippocampal

dysfunction and development of neuropsychiatric events in lupus remain elusive.

A characteristic, conserved feature of hippocampus is its neurogenic activity, which contributes to its functional plasticity.[11–13] Formation of new neurons in the adult hippocampus occurs via the neurogenic activity of adult neural stem cells (NSCs) that reside exclusively in the subgranular zone (SGZ) of dentate gyrus (DG).[14] Within this region, hippocampal NSCs (hiNSCs) can either reside in a quiescent (non-proliferating) or activated state (proliferating), the latter progressively committing to the neuronal lineage. Hippocampal neurogenesis is important for mood regulation and cognitive functions including learning and memory.[15] Accordingly, dysregulation of this process may result in behavioural deficits such as impaired cognition, depression and anxiety.[16]

In this paper, we sought to investigate the interplay between hippocampal neurogenesis and neuroinflammation using the NZB/W-F1 lupus-prone murine model. First, we validated the NZB/W-F1 as a model of diffuse human NPSLE demonstrating hippocampus-related behavioural perturbations. To elucidate the hippocampal neurogenesis, we assessed the proliferation, survival and differentiation of hiNSCs. Here, we provide evidence that IL-6 and IL-18 negatively impact hiNSC, and thus may represent therapeutic targets in NPSLE.

METHODS

Animals

New-Zealand black♀ x New-Zealand white♂ F1 mice (NZB/W-F1) spontaneously develop autoimmunity resembling human SLE.[17] NZB/OlaHsd and NZW/OlaHsd mice were purchased from Envigo.[18] Wild-type (WT) mice (C57BL/6) were used as controls. All procedures in mice were in compliance with the Greek National Law 161/91 for use of Laboratory Animals and institutional guidelines. Procedures were approved by the Greek Federal Veterinary Office (LicenseNo.1044/01-03-19). Experiments were performed in age-matched female mice (NZB/W-F1 and WT) at two different time points; 3-month-old mice (prenephritic stage; before the onset of lupus nephritis) and 6-month-old mice (nephritic stage; after the onset of lupus nephritis).

Behavioural assays

Mice were tested in a comprehensive behavioural test battery to assess possible effects on depressive-like disorders, anxiety, cognition and motor performance. The following tests were conducted in the same order for all subjects (females, NZB/W-F1, n=13 and WT, n=14) at prenephritic and nephritic stage: novel object recognition (NOR), novel object location (NOL) tasks,[19] elevated plus maze,[20] rotarod,[21] tail suspension test,[22] prepulse inhibition (PPI),[23] sociability (social novelty and social preference)[24] and sucrose preference test (SPT)[25] as previously described.[26]

Flow cytometry

Single-cell suspensions from hippocampi were generated by passing them through a 70 µm cell-strainer following tissue dissociation in RPMI-medium with DNAse-I (0.25 mg/mL, Sigma) and collagenase-D (1 mg/mL, Roche) at 37°C for 1 hour. To remove myelin, single-cell suspension was resuspended in 30% Percoll and centrifuged as previously described.[27] For staining of extracellular markers, single-cell suspensions were incubated with antibodies for 20 min at 4°C. For the staining of extracellular markers, cells were fixed and stained using the Foxp3 Staining Set (eBioscience,Cat#00-5523-00).[28] All experiments were analysed with FACS ARIA-III (BD, Bioscience). Data were analysed with FlowJo8.7 and V.X0.7 software. All antibodies are shown in online supplemental table 1.

Cell culture, TUNEL and BrdU assay

Hippocampi of 2.5-month-old female WT mice were removed. The DG was dissected from the hippocampus, digested with a papain-based solution and mechanically dissociated as previously described.[19] The dissociated cells were placed in 24-well plates with B27-medium enriched with EGF/bFGF (20 ng/mL). After 14 days in culture, the floating neurospheres were trypsin-dissociated following reformation for at least two times. Then, neurospheres were dissociated into single-cells and seeded onto poly-l-lysine coated coverslips in 24-well plate (density of 5×10^4/well). TUNEL assay and Brdu/PH3 staining were used for apoptosis and proliferation studies, respectively. For both assays, single cells remained for 24-hours on coverslips and were given the following treatments: (1) DMEM/F12 medium (in the presence or absence of EGF/bFGF, for the proliferation and apoptosis assay, respectively), (2) recombinant-murine IL-6 (100 ng/mL, immunotools) or (3) recombinant-murine IL-18 (100 ng/mL, biolegend). For the proliferation study, BrdU (10 mM) was added 2 hours before fixation. After 24 hours, cells were fixed with 4% PFA in PBS for 10 min. TUNEL assay was performed as described by the manufacturer (Promega-CatNo#G3250). For proliferation assays, cells permeabilised with 0.25% Triton X-100 in PBS followed by blocking with 10% normal goat serum in PBS. Incubations with the primary and secondary antibodies were performed for 6 hours at 4°C and for 1 hour at room-temperature respectively, in blocking solution. DAPI (1 mg/mL) was used for nuclei staining. For detection of BrdU, cells were treated with 2M HCl at 37°C for 10 min following equilibration in borate buffer (0.1M, pH8.5) for 10 min prior to primary antibody incubation. Primary and secondary antibodies are shown in online supplemental table 1.

Blood–brain barrier permeability assays

Blood–brain barrier (BBB) permeability was assessed with Blue Evans assay (EB). Mice were intravenously injected with EB dissolved in PBS in a concentration of 25 mg/kg. After 4 hours, the hippocampus was dissected from each mouse. Lung tissue from 2 mice/group were used as positive control. Dissected hippocampi were incubated with 5 mL formamide/mg tissue overnight at 60°C. Elisa reader at a wavelength of 620 nm was used to quantify the levels of EB. EB concentrations were calculate with the help of a calibration curve created from adsorption values of seven samples with predefined Evans Blue concentrations as previously described.[29]

Statistical analysis

Statistical analysis was performed taking into consideration the experimental setup using unpaired or paired Student's t-test, one-way or two-way analysis of variance (ANOVA) in GraphPad Prism V.8 software. All p values, number of samples and independent experiments are reported in the figure legends. Data are presented as mean±SD. A $p<0.05$ was considered statistical significance. Samples that were compared, were collected and analysed under the same conditions.

Detailed information for all methods including immunofluorescence, RNA-sequencing (GSE217939), qPCR and cytokines measurement are available in online supplemental material.

RESULTS

NZB/W-F1 lupus-prone mice exhibit hippocampus-related behaviour alterations recapitulating human disease

To investigate early mechanisms involved in NPSLE, we used NZB/W-F1 mice, a spontaneous lupus-prone model characterised by systemic autoimmunity. We designed a comprehensive behavioural test battery to assess depression, anxiety, cognition, motor performance/coordination and sociability. All tests were conducted in the same order for all strains both at prenephritic and nephritic-stage (figure 1A). Lupus mice were found to exhibit memory deficits at prenephritic stage based on the NOR, and spatial memory dysfunction at nephritic stage as indicated by NOL (figure 1B,C). In addition, lupus mice at both stages exhibited an anxiety-like (figure 1D) and depressive-like behaviour (figure 1E,F). Furthermore, rotarod performance showed impaired motor coordination in prenephritic and nephritic lupus mice while PPI revealed a tendency of decreased sensorimotor gating at prenephritic stage (figure 1G,H). Due to age-related hearing loss in 6-month-old WT mice, it was not possible to interpret PPI at this age (online supplemental figure 1A,B). Although no significant differences were observed in the social novelty test, lupus mice exhibited altered social recognition at both stages in the SPT (figure 1I,H). When we compared the behavioural performance of lupus mice between the two stages, we observed that lupus mice became more anxious over time (online supplemental figure 1B–G). Collectively, NZB/W-F1 mice exhibit hippocampus-related behavioural deficits early in the course of the disease, supporting the use of this model to investigate the underlying mechanisms of diffuse NPSLE.

Hippocampal neurogenesis is disrupted in NZB/W-F1 lupus

Since hippocampal behavioural deficits are often related to defective neurogenesis resulting in decreased number of neurons, we next investigated the hippocampal neurogenic activity in NZB/W-F1 lupus (figure 2A). First, we examined the neurogenic activity of hiNSCs by quantifying the cells expressing the neuronal progenitor marker doublecortin (DCX). Since the dorsal and ventral areas of DG are differentially involved in cognitive and emotional processes respectively, we analysed the expression of DCX in the two regions separately.[30] We found profound disruption (~2 fold) of hippocampal neurogenesis in prenephritic lupus compared with WT, which is preserved at the nephritic stage (figure 2B,C). Disrupted neurogenesis was also evident in the ventral area of the DG in lupus at both disease stages (online supplemental figure 2A, B). We then differentiated DCX^+ neural progenitors into early undifferentiating progenitors (without long processes) and late progenitors (with long processes) and estimated the differentiating rate of the hiNSCs (figure 2D). Importantly, we observed a decreased rate of differentiation at both stages of lupus suggesting an impaired neuronal differentiation capacity (figure 2E, online supplemental figure 2C).

To investigate the underlying mechanism of defective hippocampal neurogenesis, we determined the number and activity of radial glia-like cells (RGLs; neuronal precursors) as well as the proliferation and apoptotic capacity of hiNSCs. In prenephritic mice, there was no difference in the number of RGLs suggesting that the decreased neurogenesis observed in lupus is not due to an endogenous reduction of the hiNSCs pool (figure 2G). In contrast, the proliferation of hiNSCs was increased as evidenced by the increased number of $SOX2^+$ cells (figure 2H). We also detected a reduction in quiescent RGLs ($SOX2^-/GFAP^+$) and an increase in proliferating RGLs ($SOX2^+/GFAP^+$) (online supplemental figure 2D,E) resulting in increased proliferation rate of RGLs ($SOX2^+/GFAP^+$ RGLs to the total $GFAP^+$ RGLs) in prenephritic lupus (figure 2H,I, online supplemental figure 2F). These findings indicate increased proliferative activity of the hiNSCs at prenephritic stage. Based on comparable numbers of neuronal precursors (RGLs) and increased proliferation in prenephritic lupus mice, we reasoned that the decrease of neurogenesis may be due to increased apoptosis. Indeed, we noted increased levels of cleaved caspase-3 in the DG-isolated cells of prenephritic lupus (online supplemental figure 2G,H).

Next, we analysed the neurogenic activity at nephritic stage. Similar to the prenephritic stage, DCX^+ cells remained decreased both in dorsal and ventral regions (figure 2C, online supplemental figure 2B). Interestingly, a paradox expansion of both quiescent and activated RGLs as well as increased number of proliferating $SOX2^+$ cells was detected (figure 2G,H, D–F). However, there was no difference in proliferating rate of RGLs between lupus and WT mice (figure 2I). Thus, the increased number of RGLs at nephritic-stage may be explained by the increased activation of RGLs early in the disease course leading to increased self-renewal of neural precursors (figure 2I). However, this compensating phenomenon is not sufficient to overcome the neurogenic deficiency observed in prenephritic lupus possibly due to increased apoptosis of hiNSCs (online supplemental figure 2G,H).

Moreover, over the course of the disease, neurogenesis—although disrupted—declines with lower rate as compared with WT mice in accordance with the low exhaustion rate of RGL neuronal precursors, while the production rate of $SOX2^+$ neural progenitors remained steady (online supplemental figure 2I–K). This observation supports the increased number of RGLs detected in the late stage of the disease.

BBB disruption and immune cell trafficking orchestrate inflammatory response in lupus hippocampus at disease stage

To investigate the molecular mechanisms involved in the deregulation of hiNSCs response, we performed RNA-sequencing in the hippocampus of lupus and control mice. The analysis uncovered 578 and 721 differentially expressed genes (lupus vs WT) in prenephritic and nephritic mice, respectively (online supplemental figures 3A, B). Gene ontology analysis in the former revealed multiple enriched terms including neutrophil/myeloid leucocyte migration and leucocyte chemotaxis (online supplemental figure 3C). Similar analysis in nephritic lupus hippocampi displayed a plethora of enriched terms related to inflammatory processes including interferon signature (online supplemental figure 3D). Importantly, we observed a clear distinction between lupus and WT based on specific ‘inflammatory-associated’ genes (figure 3A,B). These data further support our findings that hippocampus-linked deficits in lupus are first evident at the prenephritic stage. Gene set enrichment analysis revealed a profound inflammatory response in lupus hippocampus at nephritic-stage compared with prenephritic stage, which is mainly mediated by peripheral inflammation (online supplemental figure 3F).

Based on the transcriptomic analysis, we asked whether the BBB is disrupted in lupus. Notably, BBB was disrupted at nephritic—but not prenephritic—stage (figure 3C, online supplemental figure 4A). We also examined immune cell trafficking from the periphery in lupus hippocampus (online supplemental figure 4B), and found a predominant myeloid response in lupus hippocampus at both stages. Specifically, an increased proportion of $CD45^+$ myeloid population were detected in lupus hippocampus, both at prenephritic and nephritic stage (figure 3D, online supplemental figure 4C), which was due to

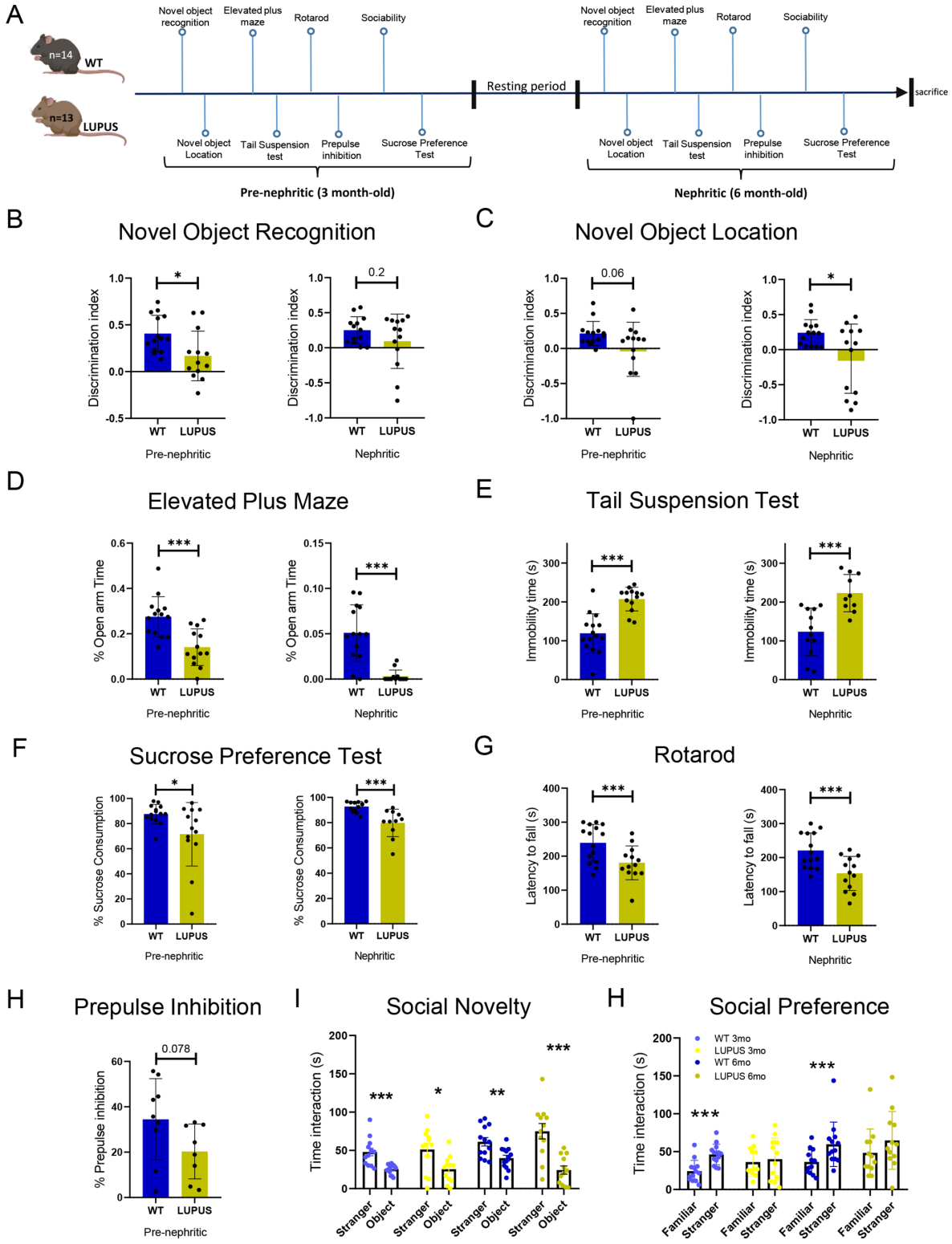

Figure 1 Hippocampus-linked behavioural alterations including impaired cognition, depressive-like behaviour and increased rates of anxiety in female NZB/W-F1 lupus mice at the prenephritic and nephritic stages of the disease. (A) Schematic representation of experimental design to assess behavioural phenotype; the same female Lupus (n=13) and WT (n=14) underwent a comprehensive behavioural test battery at 3 and 6 months of age. (B) Novel object recognition evaluates visual recognition memory; expressed as discrimination index (time spent sniffing novel object-time spent sniffing familiar object/total time spent sniffing) and (C) novel object location evaluates spatial recognition memory; expressed as discrimination index (time spent sniffing object in novel location-time spent sniffing object in familiar location/total time spent sniffing). (D) Elevated plus maze evaluates anxiety-like phenotype; expressed as time spent (%) in the open arms. (E) Tail suspension test and (F) sucrose preference test evaluate depressive-like behaviour. (G) Rotarod assesses motor performance/coordination. (H) Prepulse inhibition evaluates sensorimotor gating (n=7–8/group). (I) Social novelty and (H) Social preference evaluated with the sociability test. Lupus, NZB/W-F1 stain; WT, C57BL/6; S, seconds; Bars, mean±SD data were analysed: (B–H) Student's t-test, (I, H) two-way ANOVA, Bonferroni post-hoc test; *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; WT, wild-type.

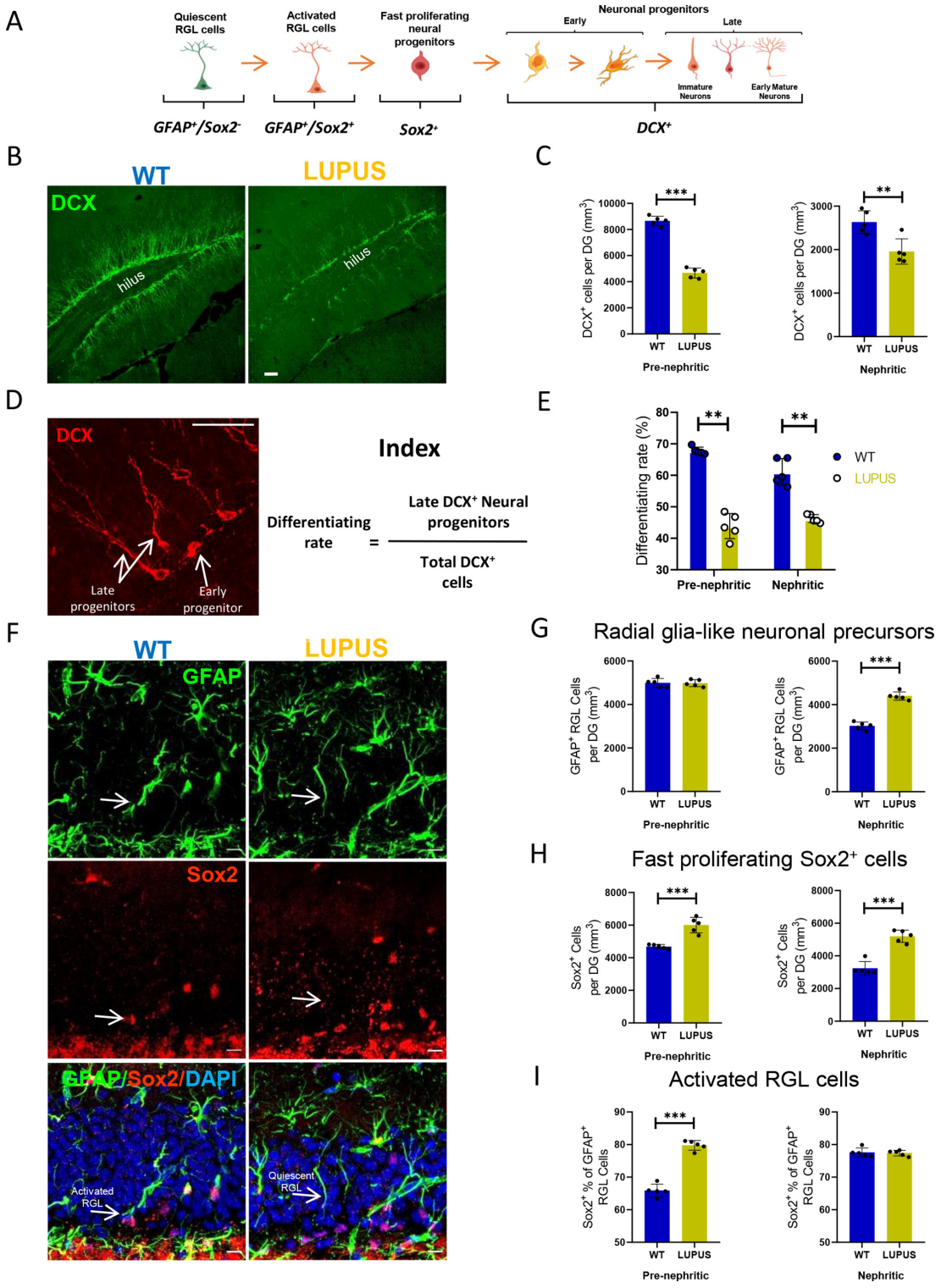

Figure 2 Hippocampal neurogenesis in NZB/W-F1 lupus mice. (A) Schematic illustration of adult neurogenesis in mice. Radial glia-like (RGL) cells express GFAP and not Sox2 under quiescent state. On activation, GFAP+RGL cells express Sox2. Fast proliferating neural progenitors at early stages of neurogenesis express Sox2. Neuronal progenitors express doublecortin (DCX) and are divided into early and late progenitors based on their morphology. (B) Representative images of immunohistochemical detection of DCX+neuronal progenitors in the DG of 3-month-old mice; Scale bar: 100 mm. (C) Quantification of DCX+cells in the DG of WT and Lupus mice at 3 and 6 months of age (n=5/group). (D) Representative image of DCX+early and late neuronal progenitors and the index used to indicate differentiating rate of neuronal progenitors. Scale bar: 10 mm. (E) Quantification of differentiating rate of DCX+neuronal progenitors in the DG of WT and Lupus mice at 3 and 6 months of age (n=5/group). (F) Representative images of immunohistochemical detection of GFAP+RGL and Sox2+cells in the DG of 3-month-old mice; Scale bar: 10 mm. (G–I) Quantification of (G) GFAP+RGL neuronal precursors, (H) Sox2+fast proliferating progenitors and (I) the proliferation rate of RGL neuronal precursors; expressed as the percentage of Sox2+/GFAP+activated RGL of total RGL neuronal precursors in the DG of WT and Lupus mice at 3 and 6 months of age (n=5/group); DAPI stains nuclei. Lupus, NZB/W-F1 stain; WT, C57BL/6; Bars, mean±SD data were analysed: C, G-I: Student's t-test, E: two-way ANOVA, Bonferroni post-hoc test; *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; DG, dentate gyrus; WT, wild-type.

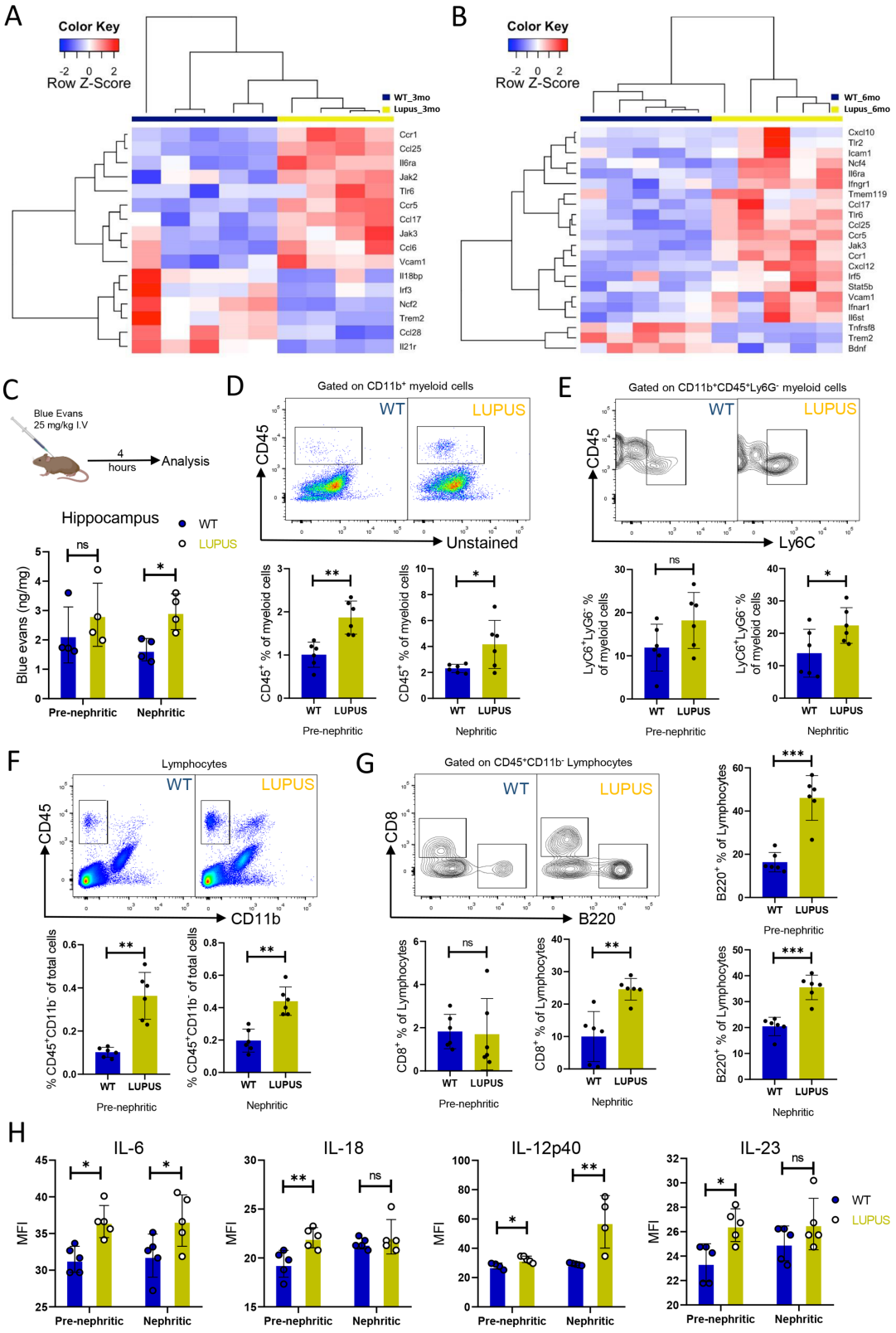

Figure 3 Increased immune cell trafficking and inflammatory response in hippocampus of NZB/W-F1 lupus mice. (A, B) Heatmaps of the expression of inflammatory associated genes in hippocampal tissue of WT and Lupus mice at (A) three and (B) 6 months of age (n=4–5/group). (C) Evans blue dye was intravenously injected followed by quantification of Evans blue in hippocampus. (D) Flow cytometry analysis; representative FACS plots and frequency of CD11b+CD45+ cells in myeloid (CD11b+) cells. (E) Flow cytometry analysis; representative FACS plots and frequency of CD11b+CD45+Ly6G-Ly6C+infiltrating monocytes in myeloid (CD11b+) cells. (F) Flow cytometry analysis; representative FACS plots and frequency of CD45+CD11b- lymphocytes in hippocampal cells. (G) Flow cytometry analysis; representative FACS plots and frequency of CD8+T cells and B220+B cells in lymphocytes (CD45+CD11b-). (H) Quantification of IL-6, IL-18, IL-12p40 and IL-23 in hippocampal tissue. All experiments (C–H) were performed in Lupus and WT mice at 3 and 6 months of age (n=4–6/group) and obtained from two independent experiments. Lupus, NZB/W-F1 stain; WT, C57BL/6; Bars, mean±SD data were analysed with Student's t-test, *p<0.05, **p<0.01, ***p<0.001. WT, wild-type.

increased $CD11b^+CD45^+LyG6^-LyC6^+$ monocytes in nephritic lupus (figure 4E, online supplemental figure 4D) and increased $CD11b^+CD45^+LyG6^+$ granulocytes in prenephritic hippocampus (online supplemental figure 4, F). We also found increased proportion of infiltrating $CD11b^-CD45^+$ lymphocytes both at prenephritic and nephritic lupus hippocampus (figure 3F), particularly $B220^+$ B-cells (figure 3G, online supplemental figure 4G,H).

Taken together, early neuropsychiatric disease is mediated by a robust myeloid response, while full-blown disease is additionally mediated by the disruption of BBB and lymphocytic cell infiltration, particularly B-cells.

Finally, we asked whether hippocampal inflammation results in increased levels of proinflammatory cytokines. Interestingly, we detected increased levels of IL-6, IL-18, IL-12p40/IL-12p70 and IL-23 in prenephritic hippocampus lupus, while IL-6, IL-12p40/IL-12p70 were elevated at nephritic stage (figure 4H, online supplemental figure 5A). We did not observe increased hippocampal levels of IL-1β, TNF-a, IL-10, TGF-b and G-CSF in lupus (online supplemental figure 5A).

Hippocampal microglia are activated towards a proinflammatory state and contribute to the local inflammation at the prenephritic stage

Our observation of intact BBB at prenephritic stage prompted us to examine the possibility that myeloid response is associated with activated microglia. We found increased expression of CD45 in hippocampal $CD11b^+$ cells, which is suggestive of microglia activation (online supplemental figure 6A–C). We also noted increased expression of the pan-microglial activation marker Iba1[31] in lupus hippocampus (figure 4A,B) especially in the areas of DG, CA1 and CA3 (online supplemental files 7A,D), at both disease stages. Finally, we verified that microglia are activated in the SGZ, GCL and hilus, subregions of the DG (figure 4C,D).

By examining directly the hippocampal microglia, we these were skewed towards an inflammatory cytokine-producing state (M1-like; indicated by increased levels of iNOS, (figure 4E), but not the anti-inflammatory phenotype (M2-like; indicated by the steady expression of arginase-1 and IL-10[32] (figure 4F, online supplemental files 5A).

Recently, microglia have emerged as antigen-presenting cells of the CNS.[33] Having detected an 'increased antigen presentation' transcriptomic signature in lupus hippocampus (online supplemental files 3D), we investigated whether microglia contribute to this signature. Specifically, hippocampal lupus microglia at both stages exhibit elevated levels of CD80, CD86 and MHC-II (figure 4G–I), suggesting that they may enhance local immune responses via antigen presentation. Microglial cells are also producers of chemokines[34] that may attract immune cells from the periphery to the CNS. To further explore this, we measured the levels of selected chemokines and found that lupus mice carry increased amounts of CCL17, CCL22 and CXCL1 (online supplemental figure 8A). To evaluate whether microglia are the main chemokine-producing cells, we measured the mRNA levels of the aforementioned chemokines from sorted lupus microglia. Of note, lupus microglia express increased mRNA levels of CCL17, CCL22 and CXCL1 (figure 4J, online supplemental figure 8B), which is correlated with the hippocampal tissue levels. These results suggest that lupus microglia release chemokines initiating immune cell trafficking.

Taken together, lupus microglial cells in the hippocampus including the DG are activated and exhibit a spectrum of pathogenetic properties including increased release of cytokines and chemokines and enhance antigen presentation.

Il-6 and IL-18 directly promote proliferation and apoptosis of hiNSCs

To further substantiate whether cytokines per se are potential regulators of hiNSCs response, we went through published available data[19] to check whether adult hiNSCs express receptors for cytokines that were found to be elevated in lupus hippocampi. hiNSCs express receptors for IL-6 and IL-18, but not for IL-12/IL-23. Therefore, we examined the effects of these particular cytokines on adult hiNSCs ex vivo with respect to proliferation and apoptosis. To this end, we isolated the DGs of 2-month-old WT mice and cultured the NSCs with growth factors until neurospheres formation. Then, we exposed the hiNSCs to either IL-6 or IL-18 for 24 hours before assessing proliferation and apoptosis (figure 5A). Interestingly, IL-6 and IL-18 exhibited similar effects on adult hNSCs by promoting proliferation (figure 5B–E) and apoptosis (figure 5F,G). Importantly, the effects of IL-6 and IL-18 on adult hiNSCs are consistent with the hiNSCs response in vivo. These data suggest that IL-6 and IL-18 may orchestrate hippocampal neurogenesis in NZB/W-F1 lupus, which is characterised by increased proliferation and decreased survival of neural progenitors resulting in disrupted neurogenesis.

DISCUSSION

Cognitive dysfunction, mood disorders and anxiety are common in NPSLE, specifically attributed to hippocampal changes. The attribution of each manifestation to lupus has important therapeutic implications since involvement of inflammatory mechanisms require the institution of immunosuppressive therapy.[2 35] In this study, we showed that NZW/B-F1 spontaneous lupus mouse recapitulates the human NPSLE and more importantly, this phenotype is associated with defective hippocampal neurogenesis due to microglial activation—in the presence of an intact BBB—leading to increased IL-6 and IL-18 production which directly induce apoptosis of adult hiNSCs (figure 6).

The pathogenesis of NPSLE remains obscure mainly due to restricted access to human tissue and limited animal models that mimic the human disease.[34–36] To date, most data on disease pathophysiology derive from studies in the MRL/lpr strain which develops an SLE-like CNS disease due to loss of Fas function although Fas-deficient humans does not develop lupus-like phenotype.[37] The neuropsychiatric disease in this model is associated with choroid plexus-infiltrating T-cell highlighting the role of choroid plexus in NPSLE pathogenesis.[38–40] Another induced model of lupus, commonly used to study cognitive impairment in SLE, involves immunisation with a peptide mimetope of DNA (DWEYS).[41] This model produces anti-DNA antibodies, termed DNRAb, that cross-react with the NMDA receptor leading to hippocampal neural cell apoptosis and cognitive decline.[5] This model has provided useful mechanistic insights for the pathogenesis of antibody-mediated damage. The production of anti-DNA starts from the age of 4–5 months in the NZB/W-F1 mice.[42] Because neuropsychiatric disease starts from the age of 3 months in NZB/W-F1 lupus in the presence of intact BBB, we infer that the anti-DNAs may contribute to underlying pathogenesis at a later stage.

In this paper, we performed a comprehensive analysis of the neuropsychiatric phenotype in the NZB/W-F1 strain[43] and report hippocampus-linked behavioural deficits including anxiety, depressive-like behaviour and impaired cognition. Importantly, these changes are detected in early stages of the disease and prior to lupus nephritis and the resultant uremia that may interfere with brain function. These findings support the use of this model in NPSLE research.

Recent studies indicate that microglia, a macrophage-like resident brain cell population is activated in lupus resulting in synaptic

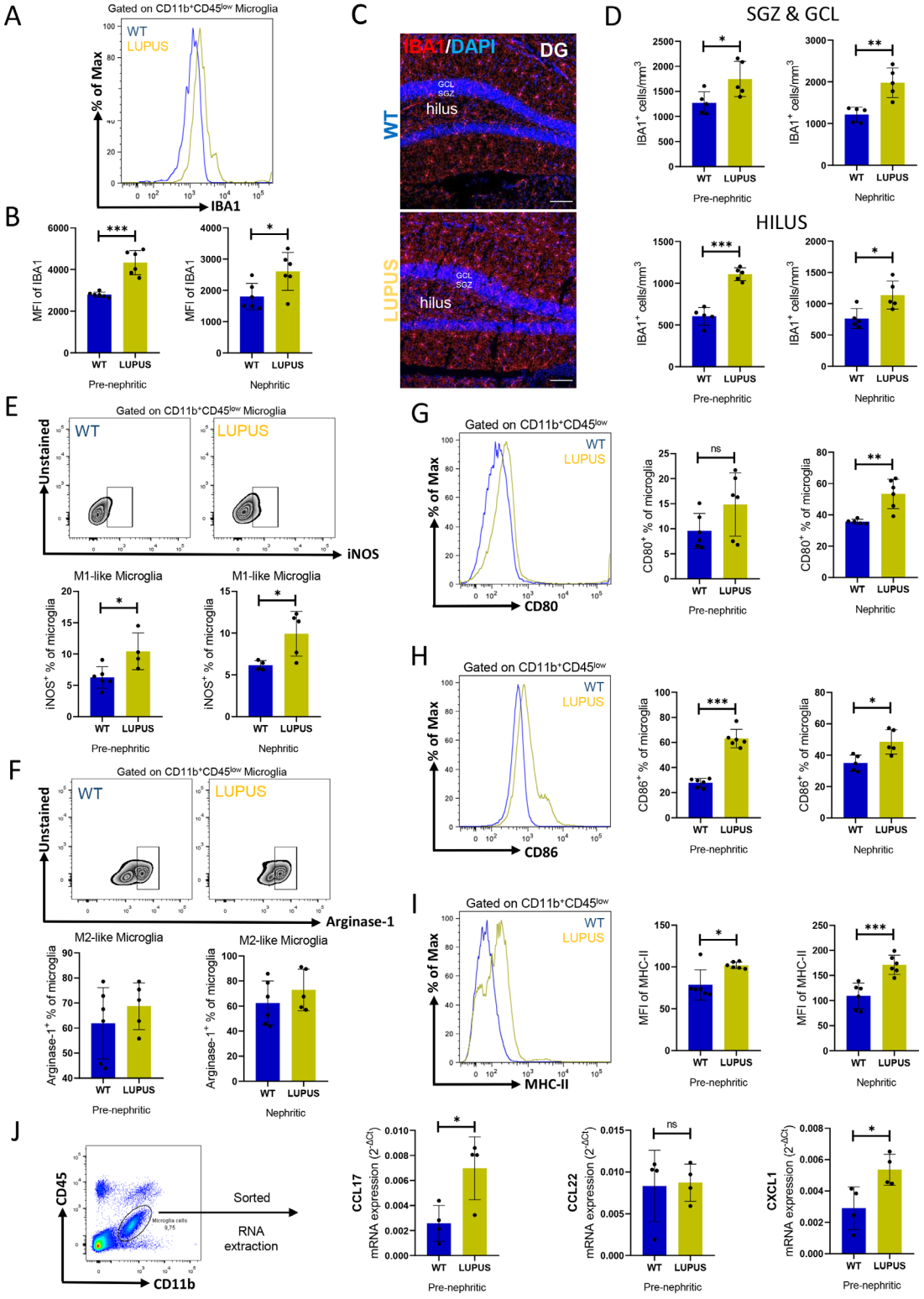

Figure 4 Activation of hippocampal microglia towards a proinflammatory state in NZB/W-F1 lupus mice. (A) Flow cytometry analysis; representative histogram and mean fluorescence intensity (MFI) of IBA1 in microglia cells (CD11b+CD45 low). (B) Representative images of immunohistochemical detection of IBA1+microglia cells in the DG of 3-month-old mice; Scale bar: 50 mm. (C) Quantification of IBA1+microglia cells in the subgranular zone (SGZ)/granule cell layer (GCL) and hilus of WT and Lupus mice at 3 and 6 months of age (n=5/group). (D) Flow cytometry analysis; representative FACS plots and frequency of iNOS+proinflammatory microglia cells (CD11b+CD45 low). (E) Flow cytometry analysis; representative FACS plots and frequency of Arginase1+anti-inflammatory microglia cells (CD11b+CD45 low). (F) Flow cytometry analysis; representative histogram and frequency of CD80+cells in microglia cells (CD11b+CD45 low). (G) Flow cytometry analysis; representative histogram and frequency of CD86+cells in microglia cells (CD11b+CD45 low). (H) Flow cytometry analysis; representative histogram and MFI of MHC-II+cells in microglia cells (CD11b+CD45 low). (I) Quantification of CCL17, CCL22 and CXCL1 mRNA levels of sorted microglia with real time RT-qPCR in 3 month-old mice. All experiments were performed in Lupus and WT mice at 3 and 6 months of age (n=4–6/group) and obtained from two independent experiments. Lupus, NZB/W-F1 stain; WT, C57BL/6; Bars, mean±SD data were analysed with Student's t-test, *p<0.05, **p<0.01, ***p<0.001.

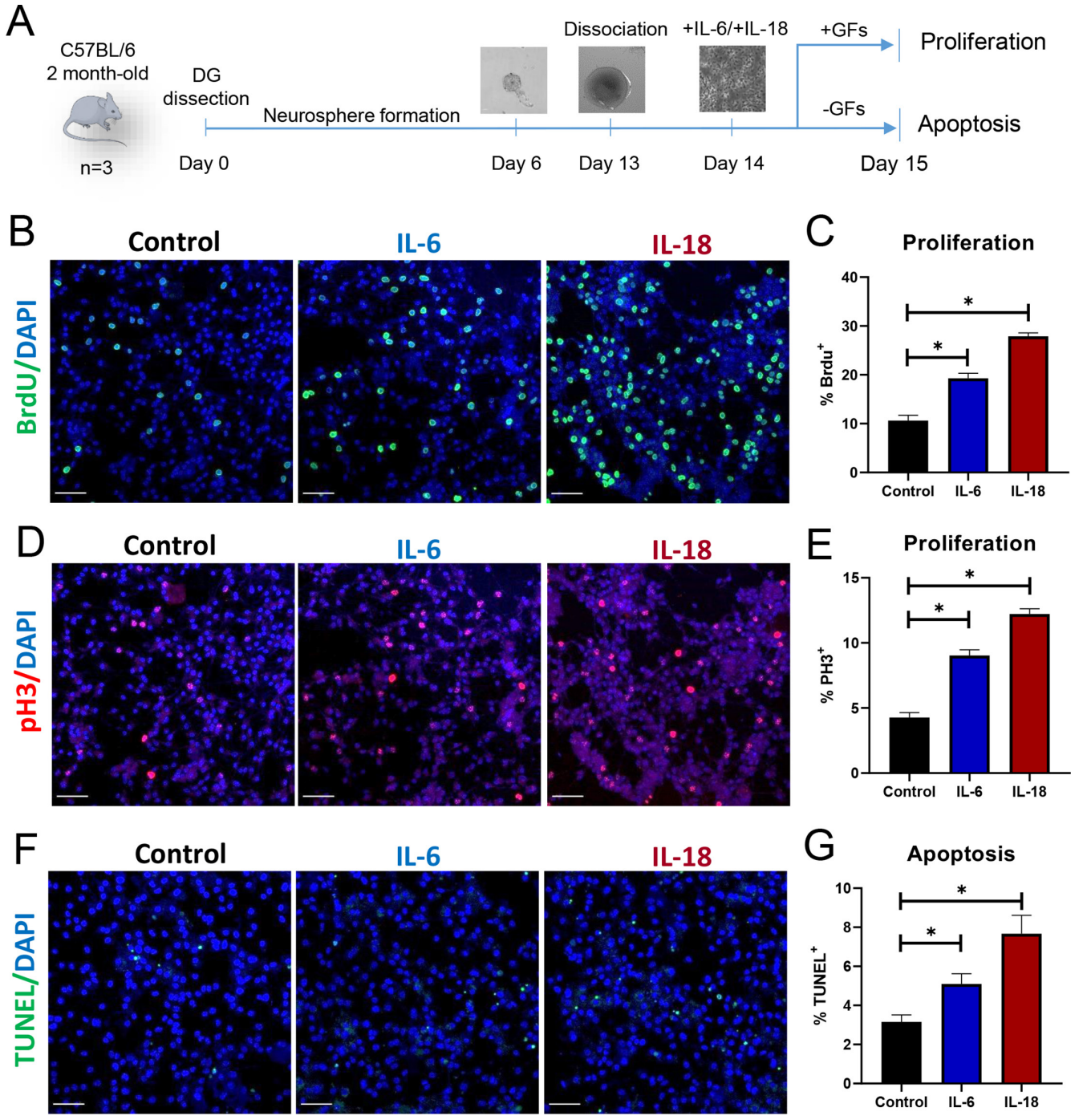

Figure 5 Interleukin-6 and Interleukin-18 directly promote proliferation and increase apoptosis of adult hiNSCs in vitro. (A) Experimental design for the assessment of direct effects of Interleukin-6 and Interleukin-18 on proliferation and survival of adult hiNSCs. (B, D, F) Representative images of adult hiNSCs in culture stained against (B) BrdU and (D) PH3 or (F) subject to apoptosis with TUNEL assay after exposure to IL-6 or IL-18. (C, E, F) Quantification of the proliferative (C, E) and apoptotic (F) effects of IL-6 and IL-18 on adult hiNSCs. Data are representative of three independent experiments. DAPI stains nuclei; Scale bar: 10 mm; bars, mean±SEM. Data were analysed with Mann-Whitney U test, *p<0.05, **p<0.01. hiNSCs, hippocampal neural stem cells.

pruning and cognitive dysfunction.[44] ACE and sTREM-1 seem to initiate microglia activation, while complement components and PI3K-AKT pathway are downstream mediators of microglia-mediated neuronal damage in NPSLE.[7 45] Moreover, activated microglia during sustained inflammation, phagocytose astrocytic end-feet and disrupt BBB permeability.[46] Here, we demonstrate for the first-time intrinsic activation of the microglia in the presence of an intact BBB at early stages of the disease. The finding of an intrinsic microglial stimulation as an early event in NPSLE is intriguing. Activated microglial cells initially migrate to the BBB to protect its integrity. However, subsequently, they are transformed into a reactive phenotype that phagocytose BBB components to initiate leakage of systemic substances into the parenchyma and cause widespread neuroinflammation.[46] Thus, activation of microglia may be a key initiating event in NPSLE. Of interest, in a recent combined genomic–genetic analysis using data from the Genotype Tissue Expression project and SLE-associated genetic polymorphisms and found that lupus susceptibility variants

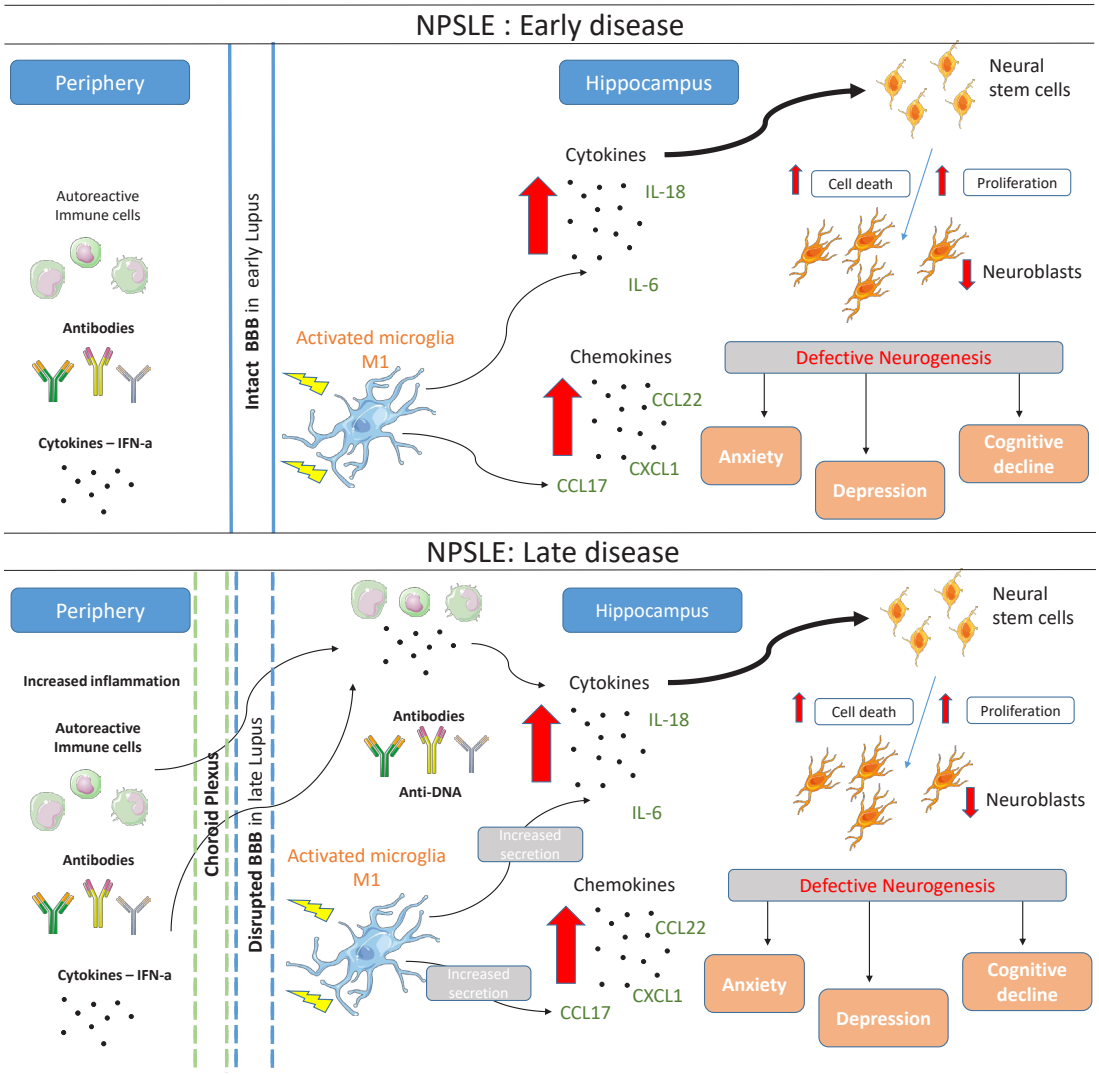

Figure 6 Proposed mechanism of early diffuse neuropsychiatric lupus. In early neuropsychiatric lupus, activation of microglia leads to the secretion of interleukin-6 and interleukin-18 which induce apoptosis to hippocampal neural stem cells (hiNSCs) leading to decreased formation of new neurons and neuropsychiatric changes. Of note, during the early stages of disease the blood–brain barrier (BBB) remains intact but activated microglia and the inflammatory cytokines may gradually impair its integrity. as the disease progresses, BBB is disrupted facilitating the entry of immune components of peripheral blood, particularly B-cells, to penetrate into the hippocampus exaggerating inflammation with locally increased levels of cytokines and an interferon signature. peripheral inflammatory mediators including interferon-α further compromise the integrity of the BBB and an interferon signature within the hippocampus becomes apparent. other pathogenetic aspects of NPSLE include the disturbance of choroid plexus and infiltration of antibodies.

(eQTLs) may regulate gene expression in the blood but also in other tissues including brain.[47] From these analyses, blood and brain emerged as the primary causal tissues in SLE[47] suggesting that brain is not only a target organ in SLE but also may represent an initiator of autoimmunity.

Inflammation of the CNS has been long recognised to lead to remodelling of the physiological activity of hiNSCs.[48] In experimental models of multiple sclerosis and inflammatory bowel disease, hippocampal neurogenesis is enhanced during the acute inflammatory phase, while progressively depleted over the course of the disease.[49–51] Defective hippocampal neurogenesis has been observed in models of induced-arthritis and BAFF-transgenic mouse; a model of systemic autoimmunity.[52–54]

To date, a thorough assessment of hippocampal neurogenesis has not been conducted in MLR/lpr lupus-prone mouse or DNRAb-induced lupus, with only one study demonstrating enhanced proliferation of hiNSCs in MLR/lpr lupus model.[55] We have characterised the hiNSCs response step by step in NZB/W-F1 strain and found that hippocampal neurogenesis is disrupted in lupus as a result of increased apoptosis. In addition, increased proliferation and self-renewal of neuronal precursors was evident that may be explained by excessive apoptosis. IL-6 and IL-18 induce apoptosis ex vivo in hiNSCs and appear to be key players of hiNSCs response in SLE. Of note, human NPSLE is associated with increased levels of IL-6 in CSF, while increased levels of serum IL-18 are linked to increased probability of NPSLE.[56 57] Of interest, in our study defective neurogenesis was observed before the onset of full-blown disease, suggesting that low-grade hippocampal neuroinflammation is an early event in lupus. From a clinical perspective, the neuropsychiatric involvement usually occurs close to diagnosis of SLE implying that disrupted neurogenesis may precede these symptoms in human SLE.

Our study highlights microglia and IL-6/IL-18 as potential therapeutic targets in NPSLE. To this end, an CSF-1R inhibitor,

which crosses the BBB and cause microglia depletion has been administrated in murine lupus with favourable CNS-related outcomes.[7] However, this drug depletes not only microglia, but also macrophages raising safety concerns.[58] Targeted therapies with monoclonal antibodies against IL-6 or IL-18 may be beneficial, yet do not cross BBB due to their size. Alternatively, JAK inhibitors can be used to target downstream cytokine-mediated pathways as they effectively penetrate the BBB.

We also detected increased levels of microglia-producing chemokines in lupus hippocampus at early stage suggesting that microglia initiate immune cell trafficking. Indeed, current evidence indicates that CCL17 regulates dendritic cell trafficking and microglia morphology towards a more inflammatory state.[59 60] Activated microglia express CCL22, which induces Th2 lymphocyte chemotaxis, suggesting that microglia-derived CCL22 drives Th2-cell recruitment to brain.[61] Consistent with our findings, CXCL1 exaggerates neuroinflammation via the attraction of neutrophils into the CNS.[62]

Type-I interferon (IFN) has emerged as central player in CNS disease causing endothelial cell damage[63] and M1-microglia activation.[64] Of note, lupus sera can induce M1 activation of brain microglia.[65] Because NZB/W-F1 is characterised by IFN-a signature at early stage,[66] we reason that early CNS IFN-response could initiate microglia activation and BBB disruption making anifrolumab a promising therapeutic target for diffuse NPSLE. However, in the NZB/NZW animal model, we did not detect a clear IFN transcriptomic signature within hippocampus at the prenephritic stage but later during full-blown disease. Whether this is a feature of other animal models of NPSLE or a unique feature of our model remains to be determined.

In conclusion, our findings support the validity of the NZW/B-F1 lupus as a model of human NPSLE with these mice exhibiting cognitive dysfunction, depression and anxiety both at early and later stages of the disease. We show that inflammation orchestrates hippocampal response in lupus mediated by both intrathecally and periphery-derived immune mediators (figure 6). IL-6 and IL-18, which are elevated in lupus hippocampus, directly induce apoptosis in hiNSCs resulting in defective neurogenesis and increased self-renewal of neuronal precursors. These disturbances underlie the behavioural phenotype of NZB/W-F1 lupus suggesting that disruption of hippocampal neurogenesis is an early event of NPSLE. Since neuropsychiatric events-especially cognitive dysfunction—in SLE may be initially subtle, inhibiting microglial activation via molecules like ACE-inhibitors or other small compounds that penetrate the BBB may be helpful at early stages.[67 68] At later stages, therapeutic strategies such as inhibition of interferon or complement components may be more helpful.

Author affiliations
[1]Center of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece
[2]School of Medicine, National and Kapodistrian University of Athens, Athens, Greece
[3]Biomedical Research Foundation of the Academy, Athens, Greece
[4]Laboratory of Autoimmunity-Inflammation, Institute of Molecular Biology and Biotechnology, Heraklion, Greece
[5]Rheumatology, Clinical Immunology and Allergy Department, Medical School University of Crete, Heraklion, Greece
[6]Medical School, University of Cyprus, Nicosia, Cyprus

Twitter Dimitrios T Boumpas @none

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ORCID iDs
Dionysis Nikolopoulos http://orcid.org/0000-0002-9894-6966
Theodora Manolakou http://orcid.org/0000-0001-5284-6655
Dimitrios T Boumpas http://orcid.org/0000-0002-9812-4671

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