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NSCs: Sentinel Cells of the Brain

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Adult neural stem cells (NSCs) have the ability to oscillate between activated and dormant states in response to extrinsic regulators. In this issue of *Cell Stem Cell*, Belenguer et al. (2020) identify a direct role for systemic TNF-α, which acts through its receptors TNFRII and TNFRI as a regulator of NSC activation and a return to quiescence, respectively.

Heterogenous pools of multipotent stem cells persist in the adult mammalian brain, wherein a balance between different cell states maintains a quiescent/dormant neural stem cell (NSC) population that preserves the endogenous brain stem cell compartment from premature exhaustion and ensures long-term maintenance.

Evidence exists of a unique quiescent (q) NSC population in the rodent subventricular zone with slow-cycling capabilities that are long-lived *in vivo* but fail to form neurospheres *in vitro* (Mich et al., 2014). This discovery has further established the foundation for primed (p) NSCs, which have the ability to become activated in response to inflammatory cytokines, including interferon (IFN)-γ (Llorens-Bobadilla et al., 2015).

When exposed to local inflammation from direct brain injury, qNSCs enter a primed state, promoting the transition into cell-cycling, active (a) NSCs (Llorens-Bobadilla et al., 2015). The characterizations of these different NSC states under homeostatic conditions, as well as the main mechanisms governing NSC state transitions, are not yet fully understood.

In this issue of *Cell Stem Cell*, Belenguer et al. (2020) describe a multilevel methodology to prospectively isolate and characterize cell populations of the subependymal zone (SEZ) lineage of the adult mouse brain and reveal that the pleiotropic effects of TNF-α on the transition between qNSCs and aNSCs are mediated by different receptors. Non-neurogenic lineage $(Lin)^-$ cells of the SEZ are fractionated and isolated into nine subsets with distinct molecular signatures and cycling behavior. Using a combination of antibody-based flow cytometry and RNA sequencing, this study suggests that pNSCs are not just a byproduct of a transient cell state, but rather a discrete NSC population that differ in their propensity to become quickly activated in response to the environment, compared to qNSCs (Belenguer et al., 2020).

Bulk RNA sequencing of the sorted $CD24^{-/low}/CD9^{high}$ NSC populations revealed that glutamate/aspartate transporter $(GLAST)^{low}$/epidermal growth factor receptor $(EGFR)^{-/low}$ pNSCs retain a unique transcriptome characterized by the expression of both cell-cycle-related genes and also quiescence-related genes. Trajectory-based pseudo-time analysis of the RNA sequencing data identified qNSCs at the beginning of the lineage with a transition to the primed state before becoming aNSCs (Figure 1A).

A single intraperitoneal (i.p.) 5-ethynyl-2 deoxyuridine (EdU) pulse combined with nuclear DNA staining labeled $EGFR^+$ cells in S/G2M, while $EGFR^{-/low}$ cells were confined to G0–G1 phases of the cell cycle.

Mice were then injected intraperitoneally for 3 consecutive days with the antimitotic agent temozolamide (TMZ) within a regeneration paradigm in which surviving qNSCs repopulate the niche (Mich et al., 2014). Flow cytometry quantification of sorted cells identified a decrease in aNSCs only. *In vitro* single-cell neurosphere assays showed early (24 h) and fast cell division with neurosphere generation by aNSCs, an absence of neurospheres by qNSCs, and delayed (7 days) neurosphere generation by pNSCs.

A method for *in vitro* fluorophore (DFFDA) dilution was also refined, and functional readouts and gene expression data were compared between DFFDA-labeled neurospheres and *ex vivo* isolated SEZ cells, as above. $DFFDA^{high}$ cells were slowly dividing and extensively self-renewing, most similar to $EGFR^{-/low}$ pNSCs. $DFFDA^{low}$ fast-cycling cells were instead short living and most similar to $EGFR^+$ aNSCs.

Therefore, slow-cycling pNSCs behave as quiescent-like cells *in vitro*, which after damage/depletion become prone to activation and contribute to repopulation of the NSC pool.

Transcriptome data analysis identified high enrichment for gene ontology (GO) term 0006954, "*Inflammatory response*," which suggests a role for inflammation in the regulation of NSC states in the SEZ niche.

The functional responses of NSCs to local and systemic inflammation have always been a matter of great debate. *In vivo*, i.p. injection of lipopolysaccharide (LPS) to model systemic inflammation increases the density of $CD68^+$ activated microglia and inhibits neurogenesis and NSC renewal in the dentate gyrus of the adult rodent hippocampus (Monje et al., 2003). *In vitro*, microglia activated with interleukin (IL)-4 or low-level IFN-γ promote NSC neurogenesis (Butovsky et al., 2006). However, IFN-γ is also able to restrict NSC cell-cycle progression to the G0 phase *in vitro* and impairs proliferation of SEZ cells *in vivo* (Pluchino et al., 2008). Furthermore, signaling through TLR2 regulates NSC proliferation, while signaling through TLR4 promotes neuronal differentiation in adult hippocampal NSCs (Rolls et al., 2007).

To understand how inflammation targets the NSC quiescence-activation balance in the SEZ, mice were injected intraperitoneally with LPS to produce inflammation-mediated inhibition of neurogenesis (Monje et al., 2003). Systemic LPS activated Toll-like receptor (TLR)-4 in niche microglia, which were largely responsible for the enhanced local expression of *Tnf in vivo*, and confirmed a previously anticipated role of TNF-α in

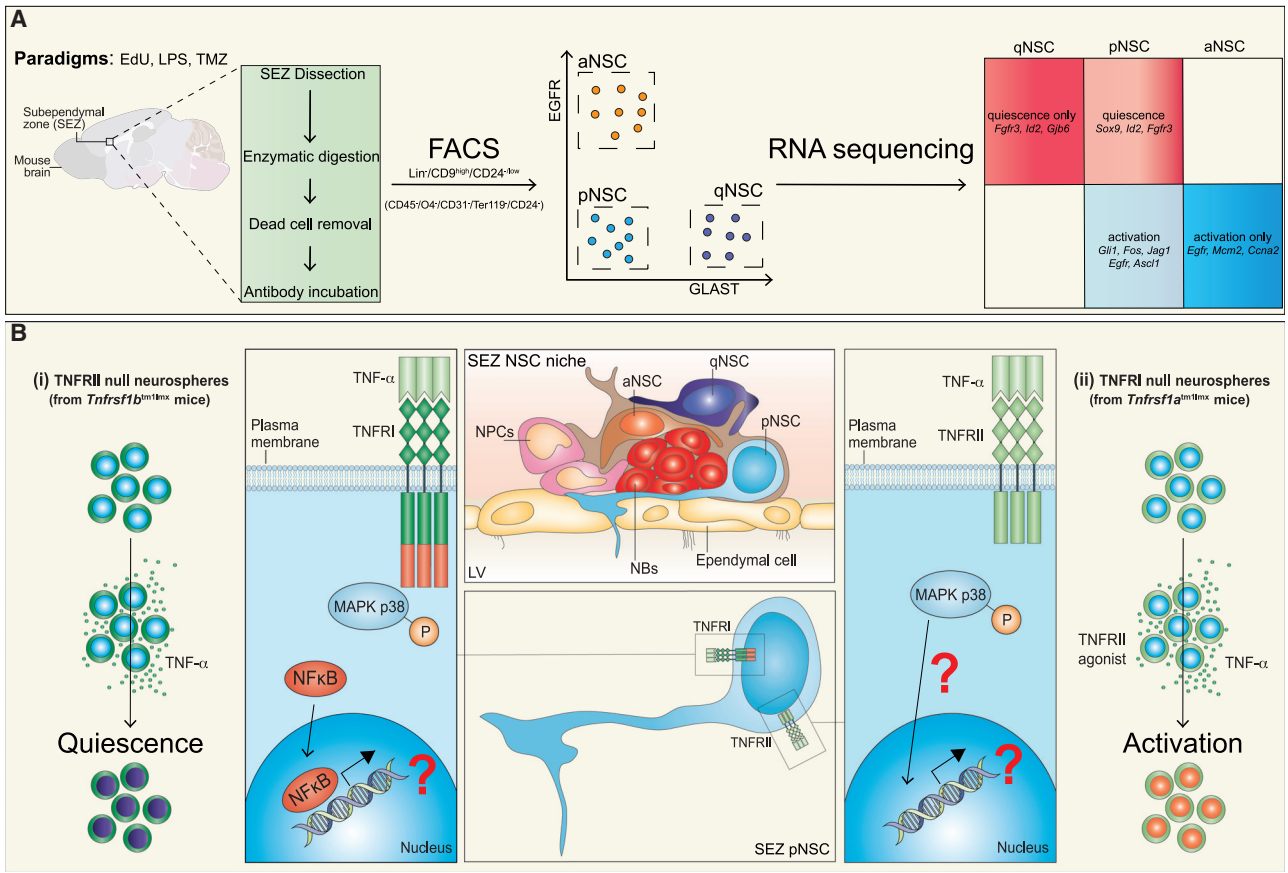

Figure 1. NSC Characterization and TNF-α Signaling Pathways

(A) NSCs were collected from the SEZ of mice and FACS was performed to sort aNSC, pNSC, and qNSC populations. Experiments were performed using a variety of paradigms, including single pulse and high-resolution pulse-chase EdU, systemic LPS, and TMZ. To remove non-neural stem cell lineage cells, cells expressing CD45, O4, CD31, Ter119, and CD24 (Lin^+ cells) were eliminated and sorting continued on $CD9^{high}/CD24^{-/low}$ cells. Further separation was used using markers for EGFR and GLAST. Using this methodology, nine cell classes were identified within the SEZ niche: neuroblasts (NBs; 68%) (NB1 were $EGFR^+$ and NB2 were $EGFR^-$); neural progenitor cells (NPCs, 6%) (NPC1 were $CD24^-$ and NPC2 were $CD24^{high}$); and NSCs (10%) (aNSCs were $GLAST^+/CD24^{-/low}/CD9^{high}/EGFR^+$, qNSCs were $GLAST^{high}/CD24^{-/low}/CD9^{high}/EGFR^-$, and pNSCs were $GLAST^{low}/CD24^{-/low}/CD9^{high}/EGFR^-$). A further $GLAST^{high}$ astrocyte subset (1.9%) and a final undefined $GLAST^-/CD24^{-/low}$ cell subset (4.4%) were also identified. Subsequent bulk RNA sequencing of the sorted populations revealed an increase in quiescence-associated genes in the qNSCs, with some upregulation in the pNSCs, along with an upregulation of activation-associated genes in the aNSCs, with some upregulation in the pNSCs.

(B) (i) Activation of TNFRI on pNSCs results in phosphorylation of MAPK p38 and stimulation of NFκB-dependent transcription, resulting in an increased expression of quiescence-related genes, inducing a quiescent state. Neurospheres isolated from (*Tnfrsf1b*^{tm1lmx}) TNFRII null mice and treated with TNF-α demonstrated a cytostatic effect, dependent on TNFRI. (ii) TNFRII activation on pNSCs induces phosphorylation of MAPK p38, resulting in a return to an activated, cycling cell state. Neurospheres from WT mice treated with a TNFRII agonist, or spheres from (*Tnfrsf1a*^{tm1lmx}) TNFRI null mice stimulated with TNF-α, showed increased self-renewal and neurosphere generation.

Abbreviations: aNSC, active NSCs; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; GLAST, glutamate/aspartate transporter; LPS, lipopolysaccharide; LV, lateral ventricle; MAPK, mitogen-activated protein kinase; NBs, neuroblasts; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NPCs; neural progenitor cells; pNSC, primed NSCs; qNSC, quiescent NSCs; SEZ, subependymal zone; TMZ, temozolamide; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

the regulation of balance between different cell states in the SEZ niche (Pluchino et al., 2008). LPS-injected mice exhibited lower numbers of cycling SEZ cells *ex vivo* but yielded more primary neurospheres *in vitro*. High-resolution EdU pulse chase studies confirmed significantly more labeled aNSCs—but not newly generated neuroblasts—and qNSCs in LPS-injected mice. Likewise, TMZ-treated mice displayed significantly higher levels of *Tnf* in SEZ cell homogenates and produced more primary neurospheres 35 days after TMZ, which were prevented by reduction of inflammation with the anti-inflammatory drugs indomethacin or minocycline.

Therefore, inflammation promotes a transient activation of $EGFR^{-/low}$ NSCs, but it has no effect on neurogenesis, under both systemic inflammation and regeneration paradigms.

Further molecular *in vitro* work with a combination of TNFRI and TNFRII null mice, activated microglia-conditioned media, and specific TNFR agonists revealed that TNF-α signals through TNFRII in a subset of slow-cycling pNSC-like cells to promote self-renewal without changing slow-cycling features. Instead, the ubiquitous TNFRI engages with TNF-α to promote cell quiescence.

LPS and high-resolution EdU pulse chase studies in TNFRI and TNFRII null mice confirmed that proliferation of pNSCs and their return to an active state in response to local TNF-α signals through TNFRII and is dependent on phosphorylation of MAPK p38. Conversely, the return to quiescence of pNSCs signals through TNFRI and is dependent on NFκB-driven transcription (Figure 1B). Finally, when analyzed in the context of TMZ-induced regeneration, TNFRII null mice showed unchanged regeneration, while isolated neurospheres exhibited significantly reduced self-renewal and multipotency; this further establishes a role for TNFRII in regulating alertness and preserving stemness when the SEZ niche is forced to regenerate under pro-inflammatory conditions.

Understanding the mechanisms regulating NSC phenotype and function in response to inflammatory signals may be useful in many different facets. In fact, persistent inflammation within the subventricular zone of the aging brain maintains NSCs in a deep quiescent state, impairing their proper activation in response to inflammation (Kalamakis et al., 2019). The recruitment of circulating T cells into the brain and local activation of microglia are both indispensable to promote neurogenesis and foster spatial learning in adulthood (Ziv et al., 2006), which suggests that systemic factors fluctuating based on disease, age, diet, and exercise have a direct and important role in the regulation of brain plasticity and regeneration. Finally, the transplantation of NSCs into laboratory animals with persistent brain inflammation promotes neuroprotection by combination of Sucnr1-dependent scavenging of the immunometabolite succinate and release of anti-inflammatory prostaglandins (Peruzzotti-Jametti et al., 2018).

In summary, Belenguer et al. describe a sorting method to study NSC states with a resolution only achieved so far by single-cell approaches, and they discover a pleiotropic role for TNF-α on NSCs, which behave as *bona fide* sentinel cells of the brain capable of both activation and return to quiescence via TNFRII and TNFRI, respectively. Further investigation of downstream signaling pathways that lead to the transcriptomic changes related to NSC activation and return to quiescence is needed.

Future work aimed at identifying druggable markers to trace these populations *in vivo* will help further elucidate the mechanisms and implications of these unique NSC states and open new opportunities to manipulate the intrinsic regeneration potential of the adult mammalian brain under brain homeostasis, (inflammatory) diseases, and aging.

DECLARATION OF INTERESTS

S.P. is co-founder and CSO at CITC Ltd. and iSTEM Therapeutics and co-founder and Non-executive Director (NED) at Asitia Therapeutics. A.N. is an advisor for iSTEM Therapeutics.

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