Acute inflammation regulates neuroregeneration through the NF-κB pathway in olfactory epithelium

Mengfei Chen[a], Randall R. Reed[b], and Andrew P. Lane[a,1]

[a]Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and [b]Center for Sensory Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205

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Adult neural stem cells/progenitor cells residing in the basal layer of the olfactory epithelium are capable of reconstituting the neuroepithelium even after severe damage. The molecular events underlying this regenerative capacity remain elusive. Here we show that the repair of neuroepithelium after lesioning is accompanied by an acute, but self-limited, inflammatory process. Attenuation of inflammatory cell recruitment and cytokine production by dexamethasone impairs proliferation of progenitor horizontal basal cells (HBCs) and subsequent neuronal differentiation. Using TNF-α receptor-deficient mice, we identify TNF-α signaling as an important contributor to this inflammatory and reparative process, mainly through TNF-α receptor 1. HBC-selective genetic ablation of RelA (p65), the transcriptional activator of the NF-κB pathway, retards inflammation and impedes proliferation at the early stages of regeneration and suggests HBCs directly participate in cross-talk between immune response and neurogenesis. Loss of RelA in the regenerating neuroepithelium perturbs the homeostasis between proliferation and apoptosis while enhancing JNK signaling. Together, our results support a model in which acute inflammation after injury initiates important regenerative signals in part through NF-κB–mediated signaling that activates neural stem cells to reconstitute the olfactory epithelium.

neuroregeneration | inflammation | olfactory stem cells | NF-κB

In the olfactory epithelium (OE), mitotically active globose basal cells (GBCs) continuously replenish olfactory sensory neurons (OSNs) lost throughout life (1–3). Wnt signaling (3, 4) and expression of transcription factors including Ascl1 (Mash1), Ngn1, and NeuroD1 (1, 5) contribute to cell fate determination and differentiation of GBCs. Horizontal basal cells (HBCs) serve as a reserve stem cell pool that is largely quiescent but becomes a major contributor after severe injury (6). The p63 transcription factor seems to be essential for HBC formation during embryogenesis and is selectively expressed in adult HBCs (7). Molecular genetic approaches have revealed a critical role for p63 in maintaining HBC self-renewal and repressing neuronal differentiation (8, 9). Two other transcription factors expressed by HBCs, Sox2 and Pax6 (10, 11), are linked to brain development and have suggested functions in regulating OSN differentiation (12, 13). New evidence based on single-cell RNA sequencing and in vivo lineage tracing has defined a detailed map of postnatal olfactory stem cell lineage trajectories and the role of Wnt signaling in driving HBCs from quiescence toward neuronal differentiation (14). Despite substantial progress, the molecular mechanisms underlying basal cell activation and neural differentiation remain incompletely understood.

Inflammation is a dynamic process involving recruitment of immune cells and secretion of proinflammatory cytokines (15). When inflammation of neural tissue is chronic there is evidence for impeded healing and repair. For example, activation of parenchymal immune cells of the brain impairs both basal and insult-induced neurogenesis in the hippocampus (16, 17). Overexpression of IL-6 by astrocytes or intracerebroventricular infusion of exogenous IL-1β also negatively regulates neurogenesis (18, 19). We have previously reported that olfactory neural progenitor/stem cell proliferation is inhibited in a mouse model of chronic sinusitis-associated inflammation generated by inducible expression of TNF-α (20). Taken together, these lines of evidence support the concept that chronic and persistent inflammation is detrimental to neurogenesis.

Although inflammation can be chronic and pathological, transient inflammation is a normal part of the host immune defense that limits infection, clears damaged cells, and initiates tissue regeneration (21, 22). Recent studies demonstrate that acute inflammation is required and sufficient to enhance proliferation of neural progenitors and subsequent neurogenesis after brain injury in zebrafish (23). In the embryonic stage, administration of IL-6 increases BrdU-labeled calretinin$^+$ neurons in the mouse olfactory bulb (24). These findings raise the possibility that inflammation directly contributes to the regulation of neurogenesis, with distinct consequences depending on the stage or intensity of the immune response.

To explore the importance of inflammation in olfactory regeneration we investigated neurogenesis after methimazole- or methyl bromide-induced olfaction lesions. We observed an acute, but self-limited, inflammatory response that coincided with early stages of neuroepithelial regeneration. Importantly, antiinflammatory treatment with dexamethasone (Dex) or global genetic deletion of TNF-α receptor 1 (TNFR1) compromised this subsequent neurogenesis. Notably, genetic elimination of the RelA subunit of the nuclear transcription factor, NF-κB, in HBCs significantly reduced neural stem cell proliferation during the early stage of regeneration. We could also examine the consequences of this HBC-selective deletion of RelA during subsequent neuronal differentiation of the HBC progeny. The absence of RelA perturbed the proliferative and apoptotic balance in the newly regenerating neuroepithelium and, in parallel, enhanced activation of JNK signaling. Therefore, this study reveals a previously unrecognized role of acute inflammation and intact NF-κB signaling in promoting olfactory regeneration.

Significance

After injury to the olfactory mucosa, neural progenitor cells residing in the basal layer are capable of completely reconstituting the neuroepithelium. The molecular events underlying this striking regenerative capacity are not fully understood. We present evidence that damage to the mouse olfactory mucosa results in a transient acute inflammatory response. Using genetic manipulations in a model of olfactory epithelial repair we demonstrate a key role of inflammation in driving the reparative activity of basal olfactory progenitor cells, in a process involving TNF-α signaling via TNF receptor 1 and the NF-κB pathway. This study, revealing cross-talk between the immune system and olfactory neural stem cells, provides insight into the endogenous activation of neuroregeneration.

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[1]To whom correspondence should be addressed. Email: alane3@jhmi.edu.

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Results

Olfactory Neuroregeneration Is Accompanied by an Acute Inflammatory Response. The methimazole-induced olfactory lesion model (25) has been widely used to study neuroregeneration in the OE. The death of OSNs in this model is considered to occur secondary to the degeneration of Bowman's glands and sustentacular cells. After lesioning, the neural stem cells residing in the basal layer are activated and reconstitute the damaged epithelium in ~2 wk. Based on the concept that inflammation is an integral component of the wound repair process (26), especially in epithelial tissue, we hypothesized that the inflammatory response actively participates in the regeneration of the OE.

We initially characterized the inflammatory response in the early stage of repair using wild-type mice. Analysis of primary proinflammatory gene expression revealed a gradual up-regulation of TNF-α, IL-1β, and IL-6 during the first week of regeneration, which subsequently declined (Fig. 1*B*). Notably, this pattern paralleled the expression of the HBC marker ΔNp63 and the neural stem cell marker Sox2 (Fig. 1*C*). Compared with nonlesioned mice, massive infiltration of $CD45^+$ inflammatory cells was detected from day 3 and became more pronounced by day 5 (Fig. 1 *D* and *E*). Costaining of CD45 and macrophage marker F4/80 revealed that ~70% of infiltrating cells were double-positive (Fig. S1*A*), suggesting that macrophages are the predominant immune cells recruited to sites of olfactory injury. ELISA analysis showed that the expression of TNF-α increased dramatically on day 3 postlesion (Fig. 1*F*). In a related but distinct model of olfactory lesioning (methyl bromide gas exposure), inflammatory cell infiltration was also observed in the lamina propria (Fig. S1*B*). From day 7, the expression of inflammatory cytokines and infiltration of immune cells decreased as tissue reepithelialization proceeded. Together, these data suggest that the acute but self-limited inflammatory response is coincident with the onset of the regeneration process upon OE injury.

Antiinflammatory Treatment Suppresses Olfactory Regeneration. To investigate the function of acute inflammation in olfactory regeneration we treated wild-type mice with the antiinflammatory corticosteroid Dex on day 2 and examined the tissue on day 3 postlesion. Dex treatment diminished the infiltration of $CD45^+$ and $F4/80^+$ inflammatory cells in a dose-dependent manner (Fig. 2*A*), as expected. Based on these results, as well as the typical dosage of corticosteroid used in the clinical treatment of inflammation, we selected a dose of 1 mg/kg Dex in subsequent studies. qPCR showed that the expression of TNF-α, IL-1β, and IL-6 mRNA in the olfactory mucosa was clearly decreased in Dex-injected mice (Fig. S2*A*). The decreased abundance of TNF-α protein paralleled mRNA changes in Dex-treated animals (Fig. 2*B*) and further confirmed the suppressed inflammatory response in lesioned tissue.

To study the impact of antiinflammatory treatment on olfactory regeneration we examined cell proliferation using BrdU on day 3 postlesion, a stage in which the inflammatory response is robustly activated (Fig. 1*D*). We observed extensive incorporation of BrdU in basal cells in PBS-treated control mice, suggesting active proliferation. Surprisingly, the number of $BrdU^+$ cells was decreased 45.1% in the Dex-treated group (Fig. 2 *C* and *D*). Furthermore, on day 5 postlesion the newly generated $Tuj1^+$ OSNs were dramatically decreased by Dex treatment (Fig. 2 *C* and *E*). Administration of Dex (1–5 mg/kg) in 3-wk-old unlesioned mice does not itself inhibit basal cell proliferation (Fig. S2*B*) or affect the number of $Tuj1^+$ OSNs (Fig. S2*C*). These observations suggest that the early immune response contributes significantly to the subsequent neuroregeneration.

TNF-α Regulates the Regeneration of the Neuroepithelium. TNF-α is one of the primary cytokines that participate in diverse inflammatory reactions. Upon binding to its two receptors, TNFR1 and TNFR2, TNF-α induces complex intracellular signaling cascades mediating inflammation and promoting cell survival or death (27). We investigated the role of TNF-α, markedly elevated after olfactory lesioning, during neuroregeneration using $TNFR1^{-/-}$, $TNFR2^{-/-}$, or double-knockout (DKO) mice. Examination of the number of $CD45^+$ immune cells, $BrdU^+$ proliferating basal cells, and $Tuj1^+$ immature neurons in unlesioned olfactory tissue from TNFR1/2-deleted mice revealed no obvious differences compared with wild-type mice (Fig. S3 *A–D*). These data suggest that deletion of

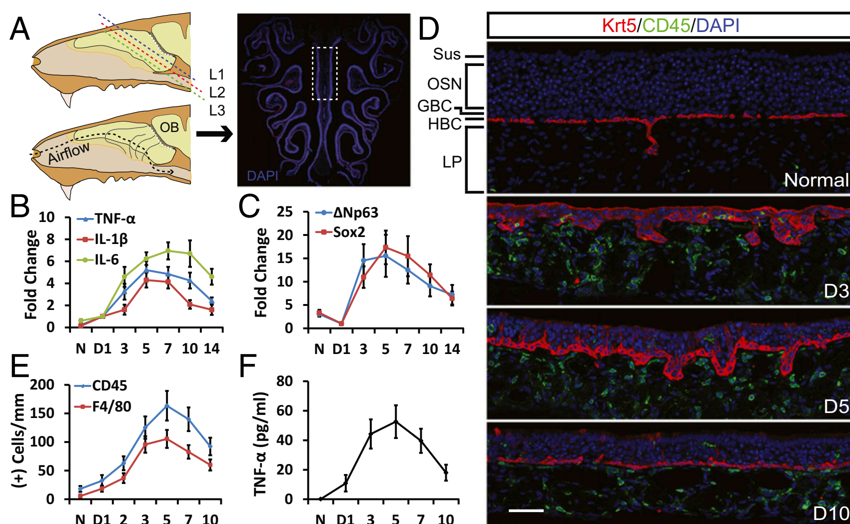

Fig. 1. OE regeneration accompanied by acute inflammatory response. (*A*) Scheme of the tissue analysis. Sagittal view of nasal cavity with (*Upper Left*) or without (*Lower Left*) septum. To avoid the variability across different animals, frozen sections were collected and examined at three consistent levels (L1–3). The boxed area was imaged at high resolution in tile scan mode and quantified. OB, olfactory bulb. (*B*) Expression of inflammatory cytokines during regeneration after olfactory lesion induced by methimazole. Quantitative RT-PCR (qPCR) results were normalized to GAPDH and levels plotted relative to day 1 ($n = 3$ mice). D, day postlesion; N, control. (*C*) qPCR analysis of ΔNp63 (the bona fide HBC marker) and Sox2 expression at different time point of regeneration. (*D*) Costaining of Krt5 and CD45 (leukocyte common antigen) in OE from normal and methimazole-treated mice. LP, lamina propria; Sus, sustentacular cell. (*E*) Quantification of $CD45^+$ and $F4/80^+$ cells in OE images. (*F*) TNF-α expression was detected in OE tissue by ELISA. Error bars represent the SEM values of three independent experiments. (Scale bar: 100 μm.)

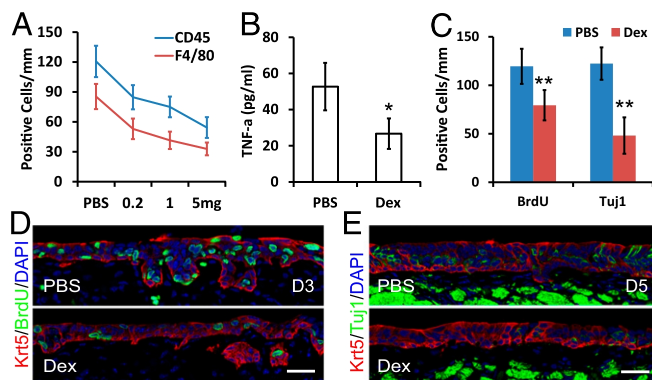

Fig. 2. Antiinflammatory corticosteroids suppress olfactory regeneration. (*A*) Quantification of $CD45^+$ and $F4/80^+$ cells in frozen sections. Wild-type mice were treated with methimazole followed by Dex i.p. with indicated dosages (milligrams per kilogram). (*B*) The expression of TNF-α in OE tissue was analyzed by ELISA. Methimazole-lesioned mice were treated with single injection of Dex (1 mg/kg) or PBS and tissue was collected on day 3 postlesion. (*C*) Quantification of $BrdU^+$ basal cells and $Tuj1^+$ cells. (*D* and *E*) Representative images of BrdU incorporation in dividing cells on day 3 (*D*) and $Tuj1^+$ newly generated OSNs on day 5 (*E*) postlesion. $^*P < 0.05$, $^{**}P < 0.01$ vs. the PBS control ($n = 3$). (Scale bars: 50 μm.)

TNF-α receptors does not affect normal immune homeostasis or stem cell proliferation in uninjured olfactory mucosa.

On day 3 postlesion, the abundance of TNF-α, IL-1β, and IL-6 mRNA in olfactory tissue was significantly decreased in $TNFR1^{-/-}$ mice (Fig. 3*A*). In agreement with previous evidence that TNFR1 mediates the majority of TNF-α responses, the expression of these cytokine mRNAs was essentially unaffected in $TNFR2^{-/-}$ mice (Fig. 3*A*). Compared with the wild-type tissue, the mRNA for transcription factors Sox2, ΔNp63, and GBC marker Ascl1 (5) were significantly down-regulated in $TNFR1^{-/-}$ but not in $TNFR2^{-/-}$ mice (Fig. 3*B*). Double knockout of both TNFR1 and TNFR2 did not lead to a further decrease of these transcription factors. Consistent with the qPCR results, TNF-α protein levels were obviously reduced in $TNFR1^{-/-}$ mice (Fig. 3*C*). The observed increase in TNF-α in DKO mice (Fig. 3*C*) may reflect the loss of feedback regulation of TNF-α expression via TNF receptor signaling (28). In addition, we detected the broad expression of TNFR1 in wild-type OE tissue (Fig. S3*E*). Costaining of p63 and TNFR1 in primary cultured HBCs further verified HBC expression of TNFR1 (Fig. S3*F*). To test whether HBCs respond to inflammatory stimulation, we treated the HBC cultures with 20 ng/mL TNF-α. qPCR results revealed that the expression of inflammatory cytokines (TNF-α and IL-6) and chemokines (RANTES and IP-10) was significantly increased in wild-type but not in $TNFR1^{-/-}$ HBCs after TNF-α stimulation (Fig. S3*G*). These observations further support a role of TNF-α signaling in the inflammation and repair process.

We next sought to address whether TNF receptor deficiency affects neuroregeneration after olfactory lesion. The number of $BrdU^+$ proliferating cells in newly formed epithelium were reduced 40.9% in $TNFR1^{-/-}$ mice (Fig. 3 *D* and *E*). Double knockout of both TNFR1 and TNFR2 caused a similar decline in $BrdU^+$ basal cells as in $TNFR1^{-/-}$ animals. No obvious difference was detected between the control and $TNFR2^{-/-}$ group. These observations suggest that TNF-α initiates regenerative signaling and promotes neural stem cell proliferation primarily through TNFR1, in agreement with previous reports that TNF-α promotes liver regeneration (29) or fracture repair (30). These results indicate that TNF-α regulates neural stem cell behavior differentially in the distinct settings of injury vs. a long-term inflammatory state, given our previous finding of repressed neurogenesis in a model of chronic TNF-α–induced olfactory inflammation (20).

NF-κB RelA Deletion in HBC Inhibits Proliferation in the Early Stage of Regeneration. The role of the NF-κB signaling pathway in regulating immune responses has been extensively studied. Proinflammatory cytokines, including TNF-α, activate NF-κB by inducing the nuclear accumulation of mainly p50-RelA (p65) heterodimers that subsequently regulate a wide spectrum of target genes involved in inflammation, antiapoptosis, and cell proliferation (31, 32). It has been shown that impairment of neurogenesis induced by chronic high-fat diet or stress is associated with NF-κB activation (33, 34). It is unclear, however, whether this NF-κB signaling is involved in the acute inflammatory response and neuroregeneration process.

To study NF-κB signaling and its importance in olfactory neuroepithelium we generated mice in which cre mediated selective deletion of exons 2–4 of RelA (35) in the HBC population and permanently labeled those cells with a td-Tomato reporter ($Krt5cre/RelA^{flox/flox}/Rosa26\text{-}stop^{flox/flox}$-td-Tomato). RelA immunostaining revealed expression in all cell types of the olfactory mucosa (Fig. S4*A*), with especially strong expression of RelA in the HBC population (Fig. S4*A*, *Upper*), reminiscent of published findings of NF-κB p50 subunit expression in the basal layer of epidermis (36). In a $RelA^{flox/flox}$ background, Krt5-cre mediated efficient elimination of RelA immunostaining and td-Tomato labeling in the HBC population (Fig. S4*A*, *Lower*). This elective ablation of RelA did not cause visible changes in HBC population in unlesioned animals. Immunohistological analysis revealed no apparent difference in the number of $CD45^+$ inflammatory cells and $Ki67^+$ proliferating cells between control and RelA-deficient mice (Fig. S4 *B* and *C*). Our observations that loss of RelA in HBCs of normal olfactory mucosa has no effect on the immune milieu and/or neural stem cell function is consistent with recent findings in RelA-deficient epidermal keratinocytes (37–39).

Given the essential role of NF-κB signaling in regulating inflammation and cell proliferation, we next asked whether the lack of RelA in HBCs affects the inflammatory response and regeneration after methimazole-induced olfactory lesioning. Compared with control mice, the number of $CD45^+$ cells in the HBC RelA-deficient mice remained largely unchanged on days 1 and 2, suggesting a

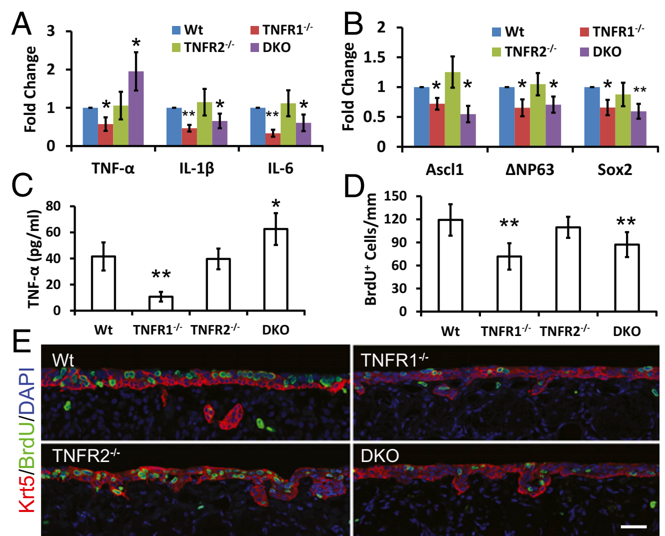

Fig. 3. Deficiency of TNF-α receptors delays regeneration of the neuroepithelium. (*A* and *B*) mRNA expression of inflammatory cytokines (*A*) and olfactory stem cell-related transcription factors (*B*) in wild-type (Wt), TNFR1, TNFR2, and DKO mice. Methimazole-lesioned OE tissue from same littermates was measured by qPCR on day 3 postlesion. (*C*) ELISA analysis of TNF-α expression in OE tissue. (*D* and *E*) BrdU incorporation in OE was detected by immunostaining (*E*), and $BrdU^+$ basal cells were quantified in *D*. $^*P < 0.05$, $^{**}P < 0.01$ against Wt ($n = 5$). (Scale bar: 50 μm.)

minor effect of HBC NF-κB signaling on inflammation at this stage. A 30% reduction of $CD45^+$ cells was observed on day 3 after lesion (Fig. S4 *D* and *E*). qPCR analysis identified significantly decreased expression of the NF-κB target gene iκBα, as well as the inflammatory cytokines, TNF-α and IL-1β, but not IL-6 in RelA-deleted mice (Fig. 4*A*).

A detailed time course analysis revealed that loss of RelA significantly reduced $Krt5^+/BrdU^+$ proliferating basal cells on days 2 and 3 after lesion (Fig. 4*B*), resulting in delayed repair. The number of $P63^+/BrdU^+$ HBCs was remarkably decreased in RelA-deleted tissue on days 1 and 2 after lesioning (Fig. 4 *C* and *D*). Given that Krt5 filaments may persist for some time after downregulation of expression of the master regulator p63 during the transition of HBC to differentiated progeny, we performed triple staining to investigate effect of RelA on this transition process. Loss of HBC RelA significantly reduced the number of $Krt5^+/P63^+/BrdU^+$ HBCs and $Krt5^+/P63^-/BrdU^+$ transitioning cells on day 2 (Fig. S5 *A* and *C*) but not day 3 postlesion (Fig. S5*B*). These data are consistent with recent findings that epidermal RelA is indispensable for keratinocyte proliferation after TPA-induced acute inflammation in skin (37).

Using td-Tomato as an HBC fate marker, we observed that ~80% of $BrdU^+$ cells are derived from labeled HBCs. Loss of RelA did not alter the proportion of td-Tomato labeled proliferating cells (Fig. S5 *D* and *F*). The number of $td\text{-}Tomato^-/BrdU^+$ cells, which are progeny of retained RelA+ GBC, was largely unchanged compared with control (Fig. S5 *E* and *F*). These data suggest that the contribution of retained GBCs, even if stimulated by inflammation, is greatly diluted by the robust expansion of HBC and their progeny progenitor cells. Together, our results support the concept that acute inflammatory response mediates repair signaling through the NF-κB pathway and contributes to neuroregeneration in the OE.

Loss of RelA Decreases Neuronal Differentiation and Increases Apoptosis. To investigate the effect of RelA absence in HBCs on later stages of regeneration we examined differentiation of olfactory precursors on day 5 postlesion. In RelA-deficient mice, costaining for Sox2 and the OSN lineage marker OE1 (40) revealed a 23.6% reduction of $OE1^+/Sox2^-$ cells compared with control mice (Fig. 5 *A* and *B*). Similarly, costaining of Sox2 and the OSN lineage marker Tuj1 showed a decreased number of $Tuj1^+/Sox2^-$ immature neurons, further verifying the decreased neuronal differentiation in RelA-deficient mice (Fig. 5 *A* and *B*). Using td-Tomato as a lineage tracing marker, we observed that 12.6% and 85.5% of differentiated $OE1^+$ OSNs are derived from labeled HBCs on days 5 and 10 postlesion, respectively (Fig. S6 *A*, *B*, and *D*). Loss of RelA decreased the percentage of $td\text{-}Tomato^+/OE1^+$ OSNs on day 5 compared with control. The td-Tomato-labeled $Tuj1^+$ immature or $OE1^+$ OSNs are comparable on day 10 postlesion (Fig. S6 *B*–*D*). This observation that loss of RelA depresses early neurogenesis in the olfactory neuroepithelium in vivo is in keeping with a previous study suggesting that NF-κB signaling is required for initiation of neural stem cell differentiation in vitro (41).

Because of the essential role of NF-κB in regulating cell survival and apoptosis (42), we asked whether loss of RelA affects the survival of newly generated OSNs. We observed a few caspase-3^+ cells distributed above the basal layer of control mice on day 10 postlesion, suggesting a process of spontaneous apoptosis in newly generated neuroepithelium. Krt5-cre–mediated RelA ablation was confirmed by anti-RelA staining (Fig. S6*E*). The number of caspase-3^+ cells was substantially increased above the basal cell layer after regeneration from RelA-deleted HBCs (Fig. 5 *C* and *D*). However, the thickness of neuroepithelium remained largely unchanged. qPCR results showed elevated expression of Ascl1 and CyclinD1 but not p63 or Sox2 in HBC RelA-deleted mice (Fig. S6*F*). BrdU incorporation analysis revealed that loss of RelA in HBCs leads to a 1.6-fold increase of proliferating basal cells (Fig. 5 *C* and *D*). Collectively, these results suggest that NF-κB signaling contributes to the homeostasis of proliferation and apoptosis in the later stages of neuroregeneration.

The proinflammatory cytokine TNF-α plays a central role within a complicated network of cytokines activating the NF-κB and JNK signaling pathways (27). Both NF-κB and JNK signaling play crucial roles in mediating the inflammatory response, and cross-talk between these two signaling pathways is recognized (43, 44). We examined the mRNA expression of iκBα (NF-κB target gene) and c-Jun (JNK target gene). Real-time PCR revealed

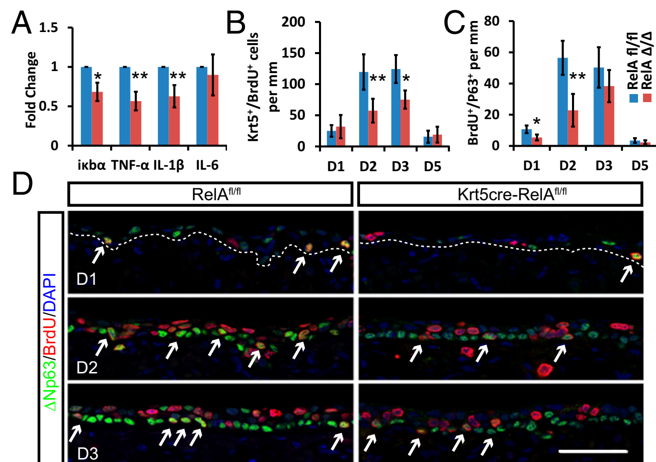

Fig. 4. RelA deletion in HBC inhibits proliferation in the early stage of regeneration. (*A*) qPCR analysis of NF-κB target gene iκBα and inflammatory cytokines on day 3 postlesion. (*B*) Quantification of $Krt5^+/BrdU^+$ basal cells. (*C* and *D*) Quantification of $P63^+/BrdU^+$ HBCs (*C*) and representative images of proliferating HBCs after methimazole lesion (*D*). Activated HBCs returned to quiescence after day 5. $^*P < 0.05$, $^{**}P < 0.01$ against control ($n = 5$). (Scale bar: 50 μm.)

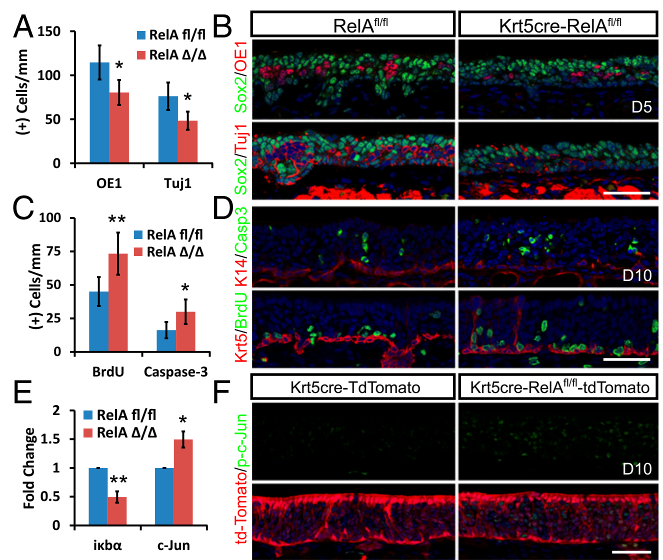

Fig. 5. Loss of RelA decreases neural differentiation and elevates apoptosis. (*A* and *B*) Immunohistochemistry analysis of newly generated neural precursors. $OE1^+/Sox2^-$ cells and $Tuj1^+/Sox2^-$ cells were counted on day 5 postlesion by methimazole. (*C* and *D*) BrdU and caspase-3 staining (*D*) revealed an increase of apoptosis and proliferation (*C*) in RelA-deleted mice. OE tissue was fixed for analysis on day 10 postlesion. (*E*) qPCR analysis of iκBα and c-Jun expression. (*F*) Immunostaining of phosphorylated c-Jun indicates JNK signaling activation in RelA-ablated OE. $^*P < 0.05$, $^{**}P < 0.01$ against control ($n = 5$). (Scale bars: 50 μm.)

decreased expression of iκBα and elevated c-Jun on day 10 postlesion (Fig. 5*E*). Of note, elevated phospho-c-Jun was observed in the regenerated neuroepithelium of RelA-ablated mice, suggesting JNK signaling activation (Fig. 5*F*). These results are consistent with published evidence that impaired NF-κB signaling enhances JNK activation (45, 46). Thus, increased proliferation and apoptosis at a later stage of regeneration in RelA-deleted mice may reflect activated JNK signaling contributing to delayed regeneration.

Discussion

Basal neural stem/progenitor cells confer a remarkable regenerative capacity to the olfactory neuroepithelium, although the underlying molecular mechanisms remain incompletely elucidated. In this study we have identified a key role for reparative inflammation in the early regenerative process and discovered an important contribution of TNF-α and NF-κB signaling in initiating basal cell neurogenesis. Acute injury of the olfactory mucosa stimulates a local inflammatory response characterized by leukocyte infiltration and cytokine production. Interference with this inflammatory process, either pharmacologically or by genetic manipulation, delays neural stem cell proliferation and neuronal differentiation. Interestingly, ablation of NF-κB signaling in the regenerating OE also results in increased neuronal apoptosis, possibly due to diminished suppression of inflammation-induced JNK activity. These results reveal a physiological function of local inflammation in the context of olfactory mucosal repair and indicate that HBCs are key cells mediating the interplay between the immune response and neurogenesis.

In mammals, the tissue response to damage occurs in three sequential stages: inflammation, new tissue formation, and remodeling (21, 26). The recruitment of monocytes to phagocytose cellular debris and prevent pathogen invasion contributes to tissue rebuilding (22). Although numerous cell types and mediators are involved in inflammation, the specific cytokines or signaling molecules directly participating in regeneration are not fully known. In the current study, we demonstrate that inflammatory cell infiltration and elevated levels of TNF-α, IL-1β, and IL-6 occur immediately after OE injury. TNF-α is a well-characterized proinflammatory cytokine that we have previously shown to elicit chronic olfactory inflammation when persistently expressed in a mouse model (20). The observation of transient TNF-α expression after acute olfactory injury led us to investigate its role in normal repair. Loss of TNFR1, but not TNFR2, significantly suppresses inflammation and reduces HBC proliferation, indicating that TNF-α contributes to the early regenerative response mainly through TNFR1. A dual relationship of TNF-α with inflammation and tissue regeneration is supported by liver injury studies (29) and fracture repair (30). The loss of both TNFR1 and TNFR2 in the olfactory lesion model does not completely block HBC proliferation, so it is clear that additional molecules and pathways are also involved.

This investigation describes a role of NF-κB in HBC regulation after injury. NF-κB is a central mediator of inflammatory and cell cycle processes, with effects in almost every cell type. The NF-κB family of transcription factors consists of five members (32): RelA/p65, c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). Many inflammatory mediators, including TNF-α, induce canonical NF-κB activation by IκBα degradation and translocation of p50-p65 dimers into the nucleus, where they bind to promoters for proinflammatory, antiapoptotic, and proliferation-associated genes (31, 47). Although not previously recognized in the OE, there is evidence in the skin that NF-κB modulates proliferation of epidermal progenitors. NF-κB activation induced by IκBα deletion (38, 48) or IKK2 overexpression in epidermal keratinocytes (49) results in skin epidermal hyperproliferation. Elevated keratinocyte proliferation in loss-of-function NF-κB mutation does not occur in a cell-autonomous fashion but occurs instead secondary to the inflammatory response (46, 49). Intriguingly, we observed that postlesion inflammatory cell infiltration and proinflammatory cytokine production were diminished in HBC RelA-deleted mice. This finding is reminiscent of the 12-*O*-tetra decanoylphorbol-13 acetate (TPA)-induced skin inflammation model, in which keratinocyte-restricted loss of RelA is associated with decreased inflammatory parameters as well as decreased keratinocyte proliferation in response to injury (37). Our observations that RelA deletion in HBC compromises the acute inflammatory response and reduces postlesion neural stem cell proliferation suggests the existence of NF-κB–mediated cross-talk between HBCs and immune cells in the early response to injury. The previously unappreciated interaction between the immune system and olfactory progenitor cells after injury is likely dynamic and comprised of multiple cell types and feedback loops. Moreover, the role of NF-κB signaling may change during the transition of bona fide HBCs into GBCs and affect the proportion of transitioning cells that return to HBCs or proceed to proliferate into differentiated olfactory cells.

NF-κB activation participates in critical regulatory pathways that oppose apoptosis (27, 50). Epithelial cell-specific deletion of IKK2 or IKKγ impairs NF-κB activity and leads to increased cell apoptosis (51, 52). In brain tissue, NF-κB activity is present in neocortex, olfactory bulbs, and hippocampus. Blocking endogenous neuronal NF-κB activity induces death of cortical neurons (53). Our finding of increased caspase-3^{+} cells in newly generated RelA-deficient OE agrees with previous reports that loss of RelA sensitizes epidermal keratinocytes to DNA damage or delayed-type hypersensitivity-induced apoptosis (37, 39). The elevated apoptosis in RelA-deficient olfactory cells we observed occurred only in the later stage of repair and coincided with increased GBC proliferation and enhanced JNK activation. Whether this JNK signaling contributes to the proliferative rebound of basal cells in RelA-deficient mice is unknown; however, delayed repair and increased spontaneous apoptosis of newborn HBC-progeny cells may trigger compensatory proliferation.

Whereas inflammation after acute injury is self-limited and important for olfactory epithelial repair, dysregulated and prolonged inflammation as seen in the human disease of chronic rhinosinusitis has very different consequences. Olfactory loss is common in patients with longstanding olfactory inflammation. Our previous investigations using a mouse model of chronic TNF-α-induced inflammation have shown widespread loss of olfactory neurons and arrest of basal cell proliferation (20). Upon removal of the inflammatory stimulus, olfactory regeneration proceeds robustly. Suppression of inflammation with corticosteroids or genetic ablation of TNF-α signaling partially reverses the block of basal cell proliferation and differentiation. This study and related ones in other neuronal tissues (16, 17) suggest a model in which the immune milieu exerts important, distinct, and potentially opposed roles at temporally separated stages and intensity of inflammation.

In summary, our study supports the concept that the acute inflammatory response, unlike the chronic inflammatory state, promotes regeneration of the olfactory neuroepithelium. Using a genetic approach we have elucidated that inflammatory cytokines, especially TNF-α, contribute to neural regeneration through the TNFR1/NF-κB pathway. In newly generated olfactory neurons, intact NF-κB activity is important for neuronal differentiation and survival. The cross-talk between NF-κB and JNK signaling may be involved in the complex relationship between inflammation and repair in olfactory neuroepithelial regeneration. Our study links the immune response to neural regeneration and provides insight into the endogenous activation of neural stem cells.

Materials and Methods

Animals. TNFR1 (54) and TNFR2 (55) knockout mice were purchased from Jackson Laboratories. The floxed RelA line (35) was kindly provided by Mollie K. Meffert, Johns Hopkins Medicine, Baltimore. Krt5-Cre transgenic line (6) in which Cre recombinase is driven by the HBC specific Krt5 promoter was crossed to RelA$^{flox/flox}$ mice to generate RelA deletion in HBCs. The Rosa26-stop$^{flox/flox}$-td-Tomato strain

was provided by Xinzhong Dong, Johns Hopkins Medicine. The animal handling procedures were approved by the Animal Care and Use Committee at the Johns Hopkins University. More details are provided in *SI Materials and Methods*.

Quantitative RT-PCR. Total RNA was extracted from isolated septum neuroepithelium using a RNeasy Mini Kit (Qiagen). Five hundred nanograms of RNA was used to synthesize the first-strand cDNA by a Omniscript Reverse Transcription Kit (Qiagen). Ten nanograms of cDNA was added to a 20-μL reaction of Taqman Fast Universal PCR Master Mix or SYBR Green Master Mix (Applied Biosystems). Real-time PCR was performed on StepOne Plus System (Applied Biosystems). Levels of mRNA expression were presented as relative expression normalized to the geometrical mean of selected reference genes. Primer sequences are listed in Tables S1 and S2.

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1. Schwob JE, et al. (2017) Stem and progenitor cells of the mammalian olfactory epithelium: Taking poietic license. *J Comp Neurol* 525:1034–1054.
2. Caggiano M, Kauer JS, Hunter DD (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: A lineage analysis using a replication-incompetent retrovirus. *Neuron* 13:339–352.
3. Chen M, et al. (2014) Wnt-responsive Lgr5^{+} globose basal cells function as multipotent olfactory epithelium progenitor cells. *J Neurosci* 34:8268–8276.
4. Wang YZ, et al. (2011) Canonical Wnt signaling promotes the proliferation and neurogenesis of peripheral olfactory stem cells during postnatal development and adult regeneration. *J Cell Sci* 124:1553–1563.
5. Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development* 129:1871–1880.
6. Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat Neurosci* 10:720–726.
7. Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. *J Neurosci* 31:8748–8759.
8. Fletcher RB, et al. (2011) p63 regulates olfactory stem cell self-renewal and differentiation. *Neuron* 72:748–759.
9. Schnittke N, et al. (2015) Transcription factor p63 controls the reserve status but not the stemness of horizontal basal cells in the olfactory epithelium. *Proc Natl Acad Sci USA* 112:E5068–E5077.
10. Davis JA, Reed RR (1996) Role of Olf-1 and Pax-6 transcription factors in neurodevelopment. *J Neurosci* 16:5082–5094.
11. Guo Z, et al. (2010) Expression of pax6 and sox2 in adult olfactory epithelium. *J Comp Neurol* 518:4395–4418.
12. Packard AI, Lin B, Schwob JE (2016) Sox2 and Pax6 play counteracting roles in regulating neurogenesis within the murine olfactory epithelium. *PLoS One* 11:e0155167.
13. Suzuki J, et al. (2015) Horizontal basal cell-specific deletion of Pax6 impedes recovery of the olfactory neuroepithelium following severe injury. *Stem Cells Dev* 24:1923–1933.
14. Fletcher RB, et al. (2017) Deconstructing olfactory stem cell trajectories at single-cell resolution. *Cell Stem Cell* 20:817–830.e8.
15. Medzhitov R (2008) Origin and physiological roles of inflammation. *Nature* 454:428–435.
16. Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302:1760–1765.
17. Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci USA* 100:13632–13637.
18. Vallières L, Campbell IL, Gage FH, Sawchenko PE (2002) Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. *J Neurosci* 22:486–492.
19. Koo JW, Duman RS (2008) IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc Natl Acad Sci USA* 105:751–756.
20. Lane AP, Turner J, May L, Reed R (2010) A genetic model of chronic rhinosinusitis-associated olfactory inflammation reveals reversible functional impairment and dramatic neuroepithelial reorganization. *J Neurosci* 30:2324–2329.
21. Karin M, Clevers H (2016) Reparative inflammation takes charge of tissue regeneration. *Nature* 529:307–315.
22. Forbes SJ, Rosenthal N (2014) Preparing the ground for tissue regeneration: From mechanism to therapy. *Nat Med* 20:857–869.
23. Kyritsis N, et al. (2012) Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science* 338:1353–1356.
24. Gallagher D, et al. (2013) Transient maternal IL-6 mediates long-lasting changes in neural stem cell pools by deregulating an endogenous self-renewal pathway. *Cell Stem Cell* 13:564–576.
25. Genter MB, Deamer NJ, Blake BL, Wesley DS, Levi PE (1995) Olfactory toxicity of methimazole: Dose-response and structure-activity studies and characterization of flavin-containing monooxygenase activity in the Long-Evans rat olfactory mucosa. *Toxicol Pathol* 23:477–486.
26. Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. *Nature* 453:314–321.
27. Brenner D, Blaser H, Mak TW (2015) Regulation of tumour necrosis factor signalling: Live or let die. *Nat Rev Immunol* 15:362–374.
28. Peschon JJ, et al. (1998) TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol* 160:943–952.
29. Yamada Y, Kirillova I, Peschon JJ, Fausto N (1997) Initiation of liver growth by tumor necrosis factor: Deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc Natl Acad Sci USA* 94:1441–1446.
30. Glass GE, et al. (2011) TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. *Proc Natl Acad Sci USA* 108:1585–1590.
31. Karin M, Greten FR (2005) NF-kappaB: Linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5:749–759.
32. Pasparakis M (2009) Regulation of tissue homeostasis by NF-kappaB signalling: Implications for inflammatory diseases. *Nat Rev Immunol* 9:778–788.
33. Li J, Tang Y, Cai D (2012) IKKβ/NF-κB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes. *Nat Cell Biol* 14:999–1012.
34. Koo JW, Russo SJ, Ferguson D, Nestler EJ, Duman RS (2010) Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc Natl Acad Sci USA* 107:2669–2674.
35. Luedde T, et al. (2008) IKK1 and IKK2 cooperate to maintain bile duct integrity in the liver. *Proc Natl Acad Sci USA* 105:9733–9738.
36. Seitz CS, Lin Q, Deng H, Khavari PA (1998) Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proc Natl Acad Sci USA* 95:2307–2312.
37. Kim C, Pasparakis M (2014) Epidermal p65/NF-κB signalling is essential for skin carcinogenesis. *EMBO Mol Med* 6:970–983.
38. Rebholz B, et al. (2007) Crosstalk between keratinocytes and adaptive immune cells in an IkappaBalpha protein-mediated inflammatory disease of the skin. *Immunity* 27:296–307.
39. Kumari S, Herzberg B, Pofahl R, Krieg T, Haase I (2014) Epidermal RelA specifically restricts contact allergen-induced inflammation and apoptosis in skin. *J Invest Dermatol* 134:2541–2550.
40. Cheng LE, Reed RR (2007) Zfp423/OAZ participates in a developmental switch during olfactory neurogenesis. *Neuron* 54:547–557.
41. Zhang Y, et al. (2012) Nuclear factor kappa B signaling initiates early differentiation of neural stem cells. *Stem Cells* 30:510–524.
42. Karin M, Lin A (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol* 3:221–227.
43. Wagner EF, Nebreda AR (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 9:537–549.
44. Tang G, et al. (2001) Inhibition of JNK activation through NF-kappaB target genes. *Nature* 414:313–317.
45. Maeda S, Kamata H, Luo JL, Leffert H, Karin M (2005) IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 121:977–990.
46. Nenci A, et al. (2006) Skin lesion development in a mouse model of incontinentia pigmenti is triggered by NEMO deficiency in epidermal keratinocytes and requires TNF signaling. *Hum Mol Genet* 15:531–542.
47. Oeckinghaus A, Hayden MS, Ghosh S (2011) Crosstalk in NF-κB signaling pathways. *Nat Immunol* 12:695–708.
48. Beg AA, Sha WC, Bronson RT, Baltimore D (1995) Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev* 9:2736–2746.
49. Page A, et al. (2010) IKKbeta leads to an inflammatory skin disease resembling interface dermatitis. *J Invest Dermatol* 130:1598–1610.
50. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr (1998) NF-kappaB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 281:1680–1683.
51. Pasparakis M, et al. (2002) TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417:861–866.
52. Nenci A, et al. (2007) Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 446:557–561.
53. Bhakar AL, et al. (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. *J Neurosci* 22:8466–8475.
54. Pfeffer K, et al. (1993) Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73:457–467.
55. Erickson SL, et al. (1994) Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* 372:560–563.