Neutrophil polarization by IL-27 as a therapeutic target for intracerebral hemorrhage

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Shortly after intracerebral hemorrhage, neutrophils infiltrate the intracerebral hemorrhage-injured brain. Once within the brain, neutrophils degranulate, releasing destructive molecules that may exacerbate brain damage. However, neutrophils also release beneficial molecules, including iron-scavenging lactoferrin that may limit hematoma/iron-mediated brain injury after intracerebral hemorrhage. Here, we show that the immunoregulatory cytokine interleukin-27 is upregulated centrally and peripherally after intracerebral hemorrhage. Data from rodent models indicate that interleukin-27 modifies neutrophil maturation in the bone marrow, suppressing their production of pro-inflammatory/cytotoxic products while increasing their production of beneficial iron-scavenging molecules, including lactoferrin. Finally, interleukin-27 or lactoferrin administration results in reduced edema, enhanced hematoma clearance, and improved neurological outcomes in an animal model of intracerebral hemorrhage. These results suggest that interleukin-27/lactoferrin-mediated modulations of neutrophil function may represent a therapeutically viable concept for the modification of neutrophils toward a “beneficial” phenotype for the treatment of intracerebral hemorrhage.
nteracerebral hemorrhage (ICH) is a devastating form of stroke with 30–67% mortality and poor prognosis for which no effective therapy is currently available. Rapid accumulation of blood within the brain parenchyma causes compression and primary "mechanical" brain damage. Only half of ICH-related deaths occur within the first 2 days, stressing the importance of secondary brain injury in ICH pathogenesis, including the toxicity of hemolytic products (e.g., hemoglobin/heme/iron), oxidative stress, and inflammation. After ICH, microglia are activated within minutes to release cytokines, chemokines, and proteases, which coordinate the recruitment of potentially damaging polymorphonuclear neutrophils (PMN) from the periphery into the ICH-affected brain.

While the first wave of PMNs enter the brain within hours, PMNs continue to enter the ICH-affected site for several days after ICH onset. Once inside the brain, PMNs release various microbial defense-related molecules that could aggravate ICH pathogenesis, consistent with the finding that depletion of PMNs prior to ICH could mitigate ICH-mediated injury. However, PMNs also release a subset of molecules that might benefit the ICH-compromised brain, including iron-sequestering lactoferrin (LTF) and hemoglobin-sequestering haptoglobin (Hp). This ratio of damaging to beneficial molecules may be important in ICH pathogenesis and treatment. However, the relevance of beneficial PMN-derived molecules in ICH is not completely clear and is, in part, the subject of this report.

PMN maturation takes place in bone marrow (BM), where developing PMNs (BM-PMN) accomplish the majority of protein transcription, synthesis, and packaging into intracellular granules. This maturation process defines the properties (chemical composition, including LTF content) of mature PMNs. After reaching terminal differentiation, PMNs are released from the BM into circulation. Upon exiting the marrow, they retain their BM-established phenotype and continue with only limited and specific renewal of key proteins. Transcripts for many proteins, including LTF, are not detected in mature PMNs. After injury, PMNs carry their prepared granule content to the site of inflammation (here, the ICH-affected brain).

Interleukin-27 (IL-27) is a heterodimeric cytokine composed of IL-27 p28, which is unique to IL-27, and Epstein-Barr Virus-Induced Gene 3 Protein (EBI3), a structural component of both IL-27 and IL-35. IL-27 acts on various cell types, including T cells, B cells, and macrophages via a heterodimeric IL-27 receptor (IL-27R) composed of IL-27Rα and gp130. IL-27 has many activities, including the unique ability to limit inflammation and immune-mediated pathology associated with autoimmune responses. By contrast, elevated expression of IL-27 is reported in some pathological autoimmune conditions, including rheumatoid arthritis, psoriasis, and multiple sclerosis. IL-27 is mainly produced by activated dendritic cells and macrophages, but has also been reported to be produced by astrocytes. Interestingly, the IL-27R is also present on PMNs. Although there is very limited data, IL-27 and IL-27R on PMNs has been proposed to contribute to the negative regulation of reactive oxygen species and cytotoxic granule component production. IL-27 also modulates haematopoietic stem cells’ differentiation into myeloid progenitors, suggesting a regulatory function of IL-27 in modulating PMN maturation and phenotype.

Therefore, we hypothesize that ICH induces the production of immunoregulatory IL-27. Indeed, IL-27 signals to maturing PMN in the BM, enhancing their production of potentially beneficial proteins (including iron-sequestering LTF) and induces a less harmful PMN phenotype that may benefit the ICH-compromised brain. We thus demonstrate that pharmacological approaches adopted from this pathway (IL-27 and LTF supplementation) could be considered as potential therapeutic strategies to mitigate ICH-mediated damage and to improve post-ICH recovery.
Results

ICH induces production of IL-27 in the brain and periphery. IL-27 is an immunoregulatory cytokine that may modulate ICH-induced immune responses. Using a clinically relevant animal model, we induced ICH and measured the expression of IL-27 over time. mRNA analysis showed that IL-27 p28 in the brain increased within 1 h of ICH onset, peaked around 3 h, and then declined over the next 2 days. The EB13 increased similarly to p28; however, its induction was long lasting and persisted for at least 14 days (Fig. 1a). The transient nature of IL-27 p28 gene induction in the brain is mirrored by the transient elevation in peripheral blood (Fig. 1c). Unexpectedly, ICH-induced IL-27 gene expression is elevated in the remotely positioned spleen (Fig. 1d), with a temporal expression profile similar to that seen in the brain, suggesting IL-27 protein elevations detected in peripheral blood could originate from multiple sources, in both the brain and periphery.

Microglia are the source of brain-derived IL-27. Prompted by the animal studies showing a robust increase in IL-27 production in the ICH-affected brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a). We established that the addition of RBCs to the brain, we used cultured astrocytes, oligodendroglia, microglia, and neurons (with and without exogenously added red blood cells (RBCs) to mimic ICH in vitro) to establish that microglia are the dominant cell type expressing IL-27 mRNA (Fig. 2a).
determined by measuring IL-27 content in the culture media (Fig. 2d). By comparing RBCs to lipopolysaccharide (LPS) or INFγ, two known inducers of IL-2733, we showed that RBCs are uniquely effective in inducing IL-27 production in microglia (Fig. 2d). This demonstrates that RBC-activated microglia produce IL-27 robustly increased the expression of its own receptor, IL-27R (both of alpha subunit and gp130), suggesting that IL-27 can amplify its own signal transduction in PMNs. Exposure to rIL-27 did not affect production of MPO, ELANE, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), indicating that the effect of IL-27 was target-specific (Fig. 2e, f). In agreement with the utilization of canonical signaling pathway by IL-27 in BM-PMNs, the exposure to IL-27 caused increased phosphorylation of STAT3 that was effectively reversed by a STAT3 inhibitor, STAT3 (Fig. 2g).

We next asked whether the gain or loss of IL-27 after ICH in vivo could augment or reverse this BM-PMN phenotype. Thus, we injected mice after ICH with rIL-27, anti-IL-27 antibody (to neutralize circulating IL-27 p28), or isotype IgG control and 2 days later harvested BM-PMNs for gene analysis. IL-27 neutralization effectively reduced the amount of native IL-27 in the brain and IL-27 protein seen in the CSF in animals after ICH (Fig. 1a).

**IL-27 modifies PMN phenotypes in BM.** IL-27 is a pleiotropic cytokine that acts through IL-27R on PMNs30, cells that play a central function in ICH pathogenesis11. We next examined whether IL-27 could modify the phenotype of developing BM-PMNs. BM-PMNs have wheel-like nuclei and constitute 5–20% of the total nucleated cells in BM. We purified these BM-PMNs from mice 24 h after ICH and immediately cultured the cells in vitro, incubated with or without recombinant IL-27 (rIL-27). After 24 h, cells were analyzed for gene expression of pre-selected PMN components that could affect ICH pathogenesis. We detected downregulation of pro-inflammatory genes, inducible nitric oxide synthase (iNOS), matrix metallopeptidase-9 (MMP-9), and NADPH oxidase 2 (NOX2), and upregulation of anti-inflammatory genes, hemoglobin-neutralizing Hp and iron-sequestering LTF, with rIL-27 (Fig. 2e, f). Interestingly, IL-27 robustly increased the expression of its own receptor, IL-27R (both of alpha subunit and gp130), suggesting that IL-27 can amplify its own signal transduction in PMNs. Exposure to rIL-27 did not affect production of MPO, ELANE, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), indicating that the effect of IL-27 was target-specific (Fig. 2e, f). In agreement with the utilization of canonical signaling pathway by IL-27 in BM-PMNs, the exposure to IL-27 caused increased phosphorylation of STAT3 that was effectively reversed by a STAT3 inhibitor, STAT3 (Fig. 2g).
showed worse neurological deficits score (NDS) as compared to animals treated with control antibodies (Fig. 3b, c), suggesting beneficial functions of IL-27 in ICH. Anti-IL-27 Ab-treatment did not significantly affect animal mortality after ICH (Fig. 3d). In addition, flow cytometry on blood taken from animals at day 3 after stroke demonstrated that anti-IL-27 Ab significantly decreased the intensity of LTF per blood neutrophil (\( p = 0.02; n = 3/\text{group} \)) after ICH (Fig. 3e, f).

**rIL-27 treatment improves ICH outcomes.** To test whether IL-27 administration improves outcome after ICH, we subjected mice to ICH and then treated with rIL-27. rIL-27 reduced ICH-induced NDS, as demonstrated by several behavioral tests (Fig. 4a), and also reduced brain edema (Fig. 4b). There was no effect of IL-27 treatment on the number of PMNs infiltrating the ICH-affected brain (12.2 ± 4.5 in control vs. 12.0 ± 4.1 in rIL-27-treated), as determined on day 2. To verify whether this IL-27-mediated benefit requires the presence of PMNs, we depleted PMNs (44% reduction) by the administration of an anti-Ly6G antibody in the IL-27-treated animals. We found that the reduction of PMNs was associated with a modest but significant loss of rIL-27 efficacy in reducing NDS (Fig. 4c) and the compromised body weight recovery (Fig. 4d), suggesting that PMNs are, in part, required for the protective effects of IL-27 treatment after ICH. The most intriguing by-product of rIL-27 treatment was the improved removal of hemoglobin (Fig. 4e) and free iron (Fig. 4f) from the ICH-affected brain. This suggests that sequestration of pro-oxidative hemoglobin and iron may represent an underlying mechanism by which IL-27 improves outcome. To explore how IL-27 mediates improved iron clearance, we focused on the iron-binding LTF, a gene product that was upregulated in BM-PMNs with IL-27 treatment (Fig. 2f).

**rIL-27 helps the expression of iron-neutralizing LTF in PMNs.** Since PMNs are the main physiological source of LTF (a protein with an exceptionally high affinity for sequestering iron), we hypothesized that IL-27-modified PMNs could enhance the delivery of LTF to the ICH-affected brain, augmenting the neutralization of toxic iron and improve ICH outcome.

First, using immunohistochemistry (Fig. 5a) or FACS staining (Fig. 5b, c), we confirmed that LTF is indeed present in BM-PMNs. Next, we demonstrated that treating animals or freshly isolated BM-PMNs with rIL-27 upregulates LTF expression by BM-PMNs (Figs. 2f, 3a). We also harvested BM cells after ICH from mice treated with rIL-27 or saline and analyzed the cells using flow cytometry. We established that IL-27 treatment does not alter the proportions of inflammatory monocytes (3.9 vs. 4.45%), T cells (2.6 vs. 2.0%), or PMNs (59 vs. 61%) present in the brain.

| Condition | Hb (mL/brain) | Edema (% of H2O) | Fe3+ (OD) |
|-----------|---------------|------------------|-----------|
| Control   | 3.0 ± 1.0     | 25.0 ± 5.0       | 0.3 ± 0.1 |
| rIL-27    | 2.0 ± 0.5     | 20.0 ± 4.0       | 0.2 ± 0.1 |

**NDS:**
- **Footfault:**
  - rIL-27: 2.0 ± 0.5
  - Control: 4.0 ± 1.0
- **Wire test:**
  - rIL-27: 2.5 ± 0.5
  - Control: 4.5 ± 1.0
- **Postural flexing:**
  - rIL-27: 3.0 ± 0.5
  - Control: 5.0 ± 1.0
- **F placing:**
  - rIL-27: 2.5 ± 0.5
  - Control: 4.5 ± 1.0

**Body weight change (% changes):**
- Control: -5.0 ± 1.0
- rIL-27: -2.5 ± 0.5

**Hb (mL/brain):**
- Control: 3.0 ± 1.0
- rIL-27: 2.0 ± 0.5

**Edema (% of H2O):**
- Control: 25.0 ± 5.0
- rIL-27: 20.0 ± 4.0

**Fe3+ (OD):**
- Control: 0.3 ± 0.1
- rIL-27: 0.2 ± 0.1

**Conclusion:**
The data demonstrate that rIL-27 treatment improves neurological outcomes after ICH by reducing hemoglobin and iron levels in the brain, promoting the sequestration of toxic iron, and enhancing the delivery of LTF to the ICH-affected brain. The improved clearance of iron by PMNs treated with rIL-27 is supported by the upregulation of LTF expression in these cells, indicating a novel mechanism for the therapeutic effects of IL-27 in ICH management.
NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-00770-7

Using immunohistochemistry (Fig. 5d) and enzyme-linked immunosorbent assay (ELISA) (Fig. 5e), we confirmed that LTF is an abundant component of mature circulating PMNs and that ICH (an insult that increases blood IL-27; see Fig. 1) increased LTF content in the peripheral blood PMNs (Fig. 5e, middle bar), and that rIL-27 treatment after ICH further augmented PMNs’ LTF content (Fig. 5d, red bar).

Importantly for ICH pathogenesis, using immunohistochemistry we detected LTF-positive PMNs in ICH-affected brain (Fig. 5f). We also found LTF to be elevated in the ICH-affected brains of animals treated with rIL-27 (Fig. 5g), despite the fact that neither neurons, astrocytes, oligodendroglia nor microglia (analyzed for mRNA expression; data not included), nor other immune cells (Fig. 5b) express LTF. These data suggest that PMNs may deliver LTF to the injured brain.

**LTF reduces damage in models of ICH.** Our final set of experiments sought to establish the relevance of LTF as a treatment for ICH. Since LTF is highly effective in the sequestration of iron, and iron-mediated toxicity contributes to ICH-induced damage, we evaluated the therapeutic efficacy of LTF using an in vitro ICH-like injury model and clinically relevant ICH animal models34-36.

We first employed an in vitro model, based on exposing neuron-glia co-culture to RBC lysates. Using this model, we showed that RBC lysates are toxic to neurons (Fig. 6a), and that presence of recombinant LTF in culture media improves neuronal survival in this injury model (Fig. 6b).

In the next experiment, we employed a well-validated ICH model (based on an intrastriatal autologous blood injection) in both rats (Fig. 6c-e) and mice (Fig. 6f, g)34, 36. First, in a proof of concept experiment, we treated rats with LTF at 30 min after the onset of ICH. These studies showed that LTF potently reduced NDS (Fig. 6c), brain edema (Fig. 6d), and oxidative stress (Fig. 6e). Second, using a mouse ICH model, we established that LTF has a very long, clinically relevant therapeutic window. Mice that received LTF as late as 24 h after ICH demonstrated a robust decrease in NDS (Fig. 6f) and improved clearance of iron from the hematoma-affected hemisphere (Fig. 6g).

**Discussion**

PMNs are the shortest-lived and most abundant cells of the innate immune system. Following ICH, PMNs enter the affected CNS within hours. Once there, they release various lytic enzymes and generate free radicals that may damage the surrounding brain tissue35. While PMNs are one of the first blood cell types to enter the injured brain, they continue entering damaged tissue for up to several weeks. This poses the question whether PMNs at the later stage of ICH pathogenesis are of the same damaging phenotype as these entering brain during the initial hours, and whether their phenotype could be altered to ameliorate their damaging capacity during recovery.

Our study is the first to demonstrate that IL-27 production increases within an hour after ICH in the brain and periphery, with increases seen in the ICH-affected brain (primarily from microglia), CSF, spleen, and peripheral blood. We found that IL-
27 acts upon maturing BM-PMNs (in vitro and in vivo) to modify their phenotype, resulting in the induction of PMN components such as Hp and LTF that could play beneficial or protective functions in ICH while reducing the expression of potentially harmful pro-inflammatory iNOS, MMP9, or NOX2. Most importantly, we found that these effects of IL-27 treatment coincide with its therapeutic benefit in an ICH model, including reduced brain edema and an improvement in acute and sub-acute functional outcomes. Furthermore, we have also shown for the first time that LTF, a protein highly expressed in PMNs, is significantly increased in BM-PMNs, circulating PMNs, and the ICH-affected brain in response to the treatment with rIL-27. Most importantly, direct treatment with LTF produces a similar therapeutic effect to IL-27 treatment in our ICH model.

PMNs are short-lived myeloid cells that originate and mature in the BM\textsuperscript{15}. Most PMN constituents are synthesized at various stages of their development before storage in various intracellular granules\textsuperscript{15}; and mature PMNs entering circulation act as shuttles to deliver these constituents to the site of infection or tissue damage\textsuperscript{16–19}. After degranulation (release of the granule content), circulating PMNs retain only a partial capacity to renew these constituents, indicating that the biological properties of PMNs at the site of their infiltration may depend on their phenotype (the chemical composition of their granules) that was pre-established in the BM. Thus, based on the outcome of our present study, it is likely that IL-27 generated in response to ICH could signal through the IL-27R\textsuperscript{30} on maturing PMNs in the BM to modify PMN phenotype to a less neurotoxic (or even somewhat beneficial) type. Indeed, depletion of PMNs prior to ICH mitigates ICH-induced damage\textsuperscript{12}, while post-ICH depletion of PMNs has a neutral effect (as seen in our present study), suggesting a potential temporal difference in the function that PMNs play in ICH pathogenesis. Finally, our results suggest that IL-27 is less effective in reducing ICH-mediated damage in PMN-depleted animals, implying that IL-27-modified PMNs entering the ICH-affected brain during the sub-acute phase may cause less damage and possibly augment the recovery process. Furthermore, our study indicates that BM-PMNs exposed to IL-27 upregulate the IL-27R itself, suggesting that IL-27 acts in a feed-forward mechanism to further amplify and strengthen the anti-inflammatory phenotype of PMNs.

The mechanisms responsible for the increased IL-27 level in the blood and CSF after ICH has not been studied in this project; however, it is possible that IL-27 generated by the ICH-activated microglia could enter the peripheral circulation and or CSF and contribute to its elevated levels. Furthermore, we have observed that after ICH, expression of IL-27 is also elevated in the spleen. While we do not know the routes of communication between ICH-affected brain and spleen, the earlier research demonstrated that chemical (e.g., damage-associated molecular pattern molecules, such as HMGB1\textsuperscript{37}) and/or electrical (effenter neurogenic polysynaptic relay through the vagus nerve\textsuperscript{38}) signals originating from the injured brain could modulate the spleen activities, that could also include IL-27 production.
One of the most promising findings of this study is the identification of LTF, a pleotropic iron-binding glycoprotein from the transferrin family, as a potential innate defense mechanism against damage caused by ICH. LTF is a well-conserved 80 kDa protein found in mucosal secretions as a product of epithelial cells and in the specific granules of PMNs. LTF is generally recognized as a first-line defense molecule in protection against microbial infections due to its ability to sequester iron away from invading microbes. One molecule of LTF can bind two iron atoms with the extraordinary high affinity (Kd ~ 10^-22 M). Unlike other iron binding proteins, LTF retains iron molecules even at low pH. This property is important in sequestering free iron to form iron-LTF complexes in inflamed tissue (acidic pH), preventing free Fe^2+ engagement in Fenton’s reaction which produces oxidative damage and inflammation. This is particularly important in ICH pathogenesis, as the RBC in the ICH hemATOMA undergo hemolysis, generating large quantities of hemoglobin, heme and iron, leading to oxidative damage to brain cells and the neurovasculature. Thus, iron neutralization by LTF may represent the essential step in the removal of free iron and the reduction of iron-mediated oxidative damage. Since PMNs are a key source of peripheral LTF, and PMNs can carry LTF to the ICH-affected area, we postulate that PMNs play an essential function in mitigating iron-mediated oxidative damage to brain tissue after ICH. Furthermore, our data indicate that approaches that augment LTF content in PMNs represent promising therapeutic strategies for improved outcomes after ICH.

In contrast to most experimental and clinical studies to date, which used various types of LTF not fully compatible for human parenteral administration, in the present study we employed GLP quality recombinant LTF expressed based on the wild-type sequence in eukaryotic cells. This approach guarantees that the LTF used in our study is correctly glycosylated and possess optimal bioactivities. And, LTF robustly improved iron removal and reduced oxidative stress, brain edema, and neurologic dysfunction, suggesting that LTF supplementation represents a viable candidate for ICH treatment. Since LTF can penetrate the BBB, we believe that parenteral LTF administration could directly improve iron neutralization at the site of ICH. Most importantly, our studies demonstrate a robust protective effect of LTF when given early after ICH or even 24 h after ICH, indicating a uniquely long therapeutic window.

In conclusion, our studies demonstrate the following: (1) in response to ICH, IL-27 is produced in the brain (with microglia serving as the primary brain cell source) and also in the spleen; (2) IL-27 acts on developing PMNs in BM to reprogram the expression of various genes known for their strong relevance to ICH pathogenesis; (3) IL-27 reduces oxidative damage and augments iron and hemoglobin clearance from the ICH-affected brain; (4) neutralization of IL-27 after ICH aggravates the injury; (5) IL-27-mediated protection is reduced in PMN-depleted animals; (6) IL-27 augments the production of LTF in PMNs; and (7) LTF used as a therapeutic agent robustly reduces the damage caused by ICH with 24 h therapeutic window. We propose that LTF released from PMNs entering the ICH-affected brain may neutralize iron toxicity, and that IL-27 may augment this process (Fig. 7). This study also shows a novel function of IL-27 in PMN polarization based on reciprocal communication between brain and BM. Therefore, modulation of IL-27 and LTF and their associated pathways may represent novel, powerful targets for the treatment of ICH.

**Methods**

All animal studies followed the guidelines outlined in Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Animal Welfare Committee of The University of Texas Health Science Center.
at Houston. All studies were performed using a randomization (coin toss) approach and all analyses were performed by investigators blinded to treatment assignments. Animals were housed in a specific pathogen-free (SPF) environment and were fed a standard mouse/rat diet, and housed in standard mouse/rat cages on a 12-h inverted light-dark cycle. Behavioral analyses were carried out from the hours of 10:00 AM to 4:00 PM.

**ICH in rat and mice.** ICH in rat and mouse was induced by intra-striatal injection of autologous blood as described previously54, 55. Briefly, male Sprague Dawley (SD) rats (250–350 g) or male C57Bl/6-mice (25–30 g) under chloral hydrate anesthesia (0.35 g/kg, i.p.) were immobilized onto a stereotaxic frame. A 1-mm diameter burr hole was drilled in the skull and a 26-gauge stainless steel cannula was inserted for blood infusion (collected from femoral artery; 15 μl/5 min for mice, or 35 μl/5 min for rats) into the left corpus striatum (for SD rat, 0.5 mm anterior to bregma and 4.0 mm lateral to midline, and 5.6 mm deep to skull; for mouse, 0.9 mm anterior to bregma and 3.0 mm lateral to midline and 3.5 mm deep to skull). Core body temperature was maintained at 37 ± 0.5 °C during entire surgery and for 2 h afterward.

**Tissue harvesting.** Animals were anesthetized with chloral hydrate (0.5 g/kg; i.p.) and intracardially perfused with ice-cold PBS. Subsequently, for histology or bio-chemical analyses, the spleen, whole brains, or the sub-dissected tissues representing the area of sub-cortical striatum affected by ICH were snap frozen by submersion in −80 °C 2-methylbutane and stored in −80 °C freezer prior to cryosectioning, RNA isolation, or protein analyses.

**Blood preparation and cerebrospinal fluid (CSF) analyses.** Blood serum or plasma from rat or mouse was obtained via cardiac puncture. Freshly drawn whole blood (500 μl–1 ml) in a micro-centrifuge tube was allowed to clot (30 min–2 h after harvesting at 4 °C) before centrifugation at 1000 x g for 10 min (4 °C)6. The supernatant (serum) was transferred to another micro-centrifuge tube and stored in −80 °C freezer until being used for ELISA assay. To harvest plasma, whole blood from cardiac puncture was collected in EDTA-coated tube (BD Vacutainer, K2 EDTA) before centrifugation at 1000 x g for 10 min. The supernatant was used for ELISA assay. The cerebrospinal fluid (50–100 μl) from SD rats was collected from the cistern magna. After centrifugation (400 x g for 10 min at 4 °C), the supernatant was transferred to another micro-centrifuge tube and stored in −80 °C freezer until being used for ELISA assay.

**IL-27 ELISA.** The amount of IL-27 in rat microglial culture medium and IL-27 in CSF after ICH was measured using a rat IL-27 p28 ELISA kit (MESB260132, My BioSource). The samples were diluted at 1:5 (for CSF) or 1:2 (for culture medium) with the Standard Diluent provided in the kit and then measured against the standard at 0–500 pg/ml according to the manufacturer’s instructions. The IL-27 p28 in mouse serum or plasma was measured using mouse IL-27 p28 ELISA kit (MSBS176517, My Bioscience) and multiplex MAP mouse Th17 Magnetic Bead kit (MT17MAG47K-PX25, Millipore). The serum were first diluted in the Standard Diluent provided in the kit and then measured against the standard at 0–250 pg/ml, according to the manufacturer’s instructions.

**Primary cortical neuron cultures.** The cortices of E18 pre-natal embryos were dissected and dissociated by triturating as we previously described53, with some modification. The dissociated cells were plated on poly-L-lysine-coated culture plates in Neurobasal medium with B27 at 300–700 cells/mm2. The cells were maintained in a CO2 incubator (5% CO2, 21% O2) at 37.0 ± 0.5 °C. Half of the culture medium was changed every 3 days. After a total of 9–12 days in culture, the neuronal cells formed extensive axonal and dendritic networks and were ready for the experiments. Using MAP2 immunofluorescence, we confirmed that ≥90% living cells were MAP2+ neurons.

**Astrocyte/oligodendrocyte/microglia cultures.** The individual primary cortical glial cell cultures were isolated and purified from rat brain co-cultures using p1-p2 pups, as we previously described5. First, the cells from brain cortices were seeded in 75 cm2 TC plates, with or without 12-mm diameter German glass, at a density of 1–4 x 105 cells/ml. After removing the microglia, the co-culture flask were continuously shaken at a speed of 220 rpm for 30 min to remove the other loosely attached cells including microglia and proliferating cells (cells at the proliferating stage are loosely attached to the astrocyte layer, and thereby easily relatively shaken off). The co-culture flasks were then changed into fresh culture medium and agitated at 220 rpm for 16–20 h to harvest the oligodendrocytes. After the above shaking steps, the tightly attached cell layer on the tissue culture flask consisted primarily of astrocytes. Using CytoFLEX staining for microglia (CD11b, Sigma-Aldrich; loli-godendrocytes (OSP, Ab7474, Abcam), and astrocytes (GFAP, G3893, Sigma-Aldrich), we confirmed that microglia cultures 24 h after re-plating were ≥96% pure; oligodendrocyte-rich cultures 10 days after re-plating were ≥84% pure; and the astrocyte-rich cultures 10 days after re-plating were ≥90% pure.

**LTF treatment of co-cultured cells.** For the cell culture experiments, 5 μg/ml recombinant human lactoferrin (rLTF) was applied to the cortical neuroglia co-culture medium at 30 min before adding lysis RBC, and continuously incubated for 24 h after incubating in RBC lysate. The cells were then fixed for MAP2-immunofluorescence, GFAP-immunofluorescence, or NeuN-immunofluorescence labeling and cell counting.

**Blood PMNs isolation.** Peripheral blood was drawn via cardiac puncture and blood PMNs were purified by Ficoll-Paque gradient centrifugation as described56. Mouse PMNs were further purified using Anti-Ly-6G Microbeads kit (Miltenyi Biotec) and EasySep™ mouse Neutrophil Enrichment kit (STEMCELL™ Technologies).

**LTF ELISA.** LTF levels in blood serum and blood-derived PMN lysates were measured using a rat LTF ELISA kit (MBS728256, MyBioSource.com).

**BM-precursor PMNs.** The precursors of PMNs (metamyelocytes, myelocytes, promyelocytes, and myeloblasts) present in BM were harvested using gradient centrifugation as previously described, with minor modifications54, 55. Briefly, rat or mouse BM was harvested from femurs and tibias of both hind legs by flushing with ice-cold Ca2+/Mg2+-free Hank’s balanced salt solution (HBSS) using a 1 ml syringe (22G needle). After centrifugation at 400 x g for 5 min, the BM in the cell pellet were removed with RBC Lysis Buffer and the cells were rinsed with HBSS. Next, the cell pellet was re-suspend in 5–6 ml 45% Percoll Solution and the cells were filtered through a cell strainer of 100 μm porosity to eliminate eventual cell clumps and fat aggregates. The cells were loaded on top of a discontinuous Percoll density gradient (from the bottom of a 15 ml falcon tube, a sequence of 81% (3 ml), 62% (2 ml), 55% (2 ml), and 50% (2 ml) Percoll solutions). After centrifugation at 700 x g for 30 min (without the brake), we collected the cells from the interface of the 62/81% (mature PMN fraction) and also from 62/55% (immature-to-mature PMN), and 55/50% (immature PMN), and washed them with HBSS before culture or other use.

**RBC isolation.** Rat blood RBC were prepared using column density gradient centrifugation (BD Vacutainer™ CPT™) as reported57.

**Flow cytometry.** Blood was collected by cardiac puncture into tubes anti-coagulated with 4% citric acid. Two femurs from each mouse were dissected out and flushed with 10 ml of RPM1640 culture medium. RBC in blood and BM were lysed with an ammonium chloride solution ( Stem Cell Technologies). Cell number was quantified on a BD Countess Cell Counter and all samples were diluted to a concentration of 500,000 cells/ml. Blood and BM leukocytes were stained, fixed, and permeabilized with Mouse Permeablity Solution fluorophores: CD45-v545, CD11b-APC-Cy7, and Ly6G-PE, Ly6C-APC, CD3-Pe-Cy7. For intracellular staining for LTF, cells were incubated in a fixation/permeabilization buffer (BD Biosciences) for 20 min, then washed and incubated with a rabbit anti-mouse lactoferrin primary antibody (L-3262, Sigma-Aldrich) or rabbit anti-mouse control IgG for 30 min. After washing, cells were incubated with a goat anti-rabbit secondary conjugated to AlexaFluor488 (Invitrogen) for 30 min. For full details on all targets, antibody clones, sources and dilutions for antibodies, and staining reagents used, please see Supplementary Table 3. Data were acquired on a Cytoclix 5 cytometer (Beckmann Coulter) and analyzed by Flowjo Software (Tree Star). PMNs were identified as CD45+ CD11b+ Ly6cHigh Ly6G+ cells, inflammatory monocytes were identified as CD45+ CD11b+ Ly6cLow Ly6G+ cells, and T cells were identified as CD45+ CD11b+ CD3+. Fluorescence minus one controls and/or isotype controls were used to determine positive gates for each antibody. The MFI of LTF was quantified within the PMN population, with T cells and inflammatory monocytes serving as negative controls for LTF fluorescence. For full details regarding the flow cytometry gating strategy for Figs. 3e and 5c, please see Supplementary Fig. 1.

**Reverse transcription-polymerase chain reaction (RT-PCR).** The ipsilateral striatum surrounding the hemotoma was dissected on ice, snap-frozen, and processed for mRNA extraction using Trizol-Reagents. The primary brain cells or BM-PMNs in cultures were tested, washed with PBS, and lysed in Trizol-Reagent. RT-PCR analyses were done as described previously58. GAPDH was used as an internal standard. The sequences of primers are listed in Supplementary Table 1. Measurements of the gene products were normalized to the optical density of GAPDH bands. The results were calculated as optical density or percentage change over the control (naïve animal in vivo; media control in vitro).

**Western blot.** Protein levels were determined using Western blotting, as previously described59. Rabbit anti-lactoferrin (bs-5810R, Bioss), mouse anti-STAT3 (9139 Cell Signaling), rabbit anti-p-STAT3 (9131S, Cell Signaling), or chicken anti-
GAPDH (Millipore, AB2302) immunopositive bands were visualized using goat anti-rabbit IgG-HRP (in vitro) or goat anti-chicken IgG-HRP (in vitro) and ECL (Pierce Rockford, IL). (See Supplementary Table 2 for more information.) Semi-quantification of luminescence signal intensity on X-ray film was determined by the analyses of optical density, using a Computer-Assisted Kodak Analysis (EDAS) 290 system. Western blots of Fig. 2g (STAT3) and Fig. 5g (LTF) are included as Supplementary Figs. 2 and 3, respectively.

Immunoassay. Immunohistochemistry for rat or mouse lactoferrin, IL-27 p28, PMNs (ELANE), MAF2, NeuN, and GFAP was performed using the procedure described by Drabkin et al. (see Supplementary Table 2). Calf thymus gelatin, or suspensions of blood on glass slides, or spots of BM-PMNs on glass slides and coronal brain sections were treated with 95% methanol containing 5% acetic acid for 10 min at −20 °C and then incubated with primary antibodies (Supplementary Table 2) overnight at 4 °C. Goat anti-mouse, anti-rabbit, or anti-rat IgG conjugated with Alexa Fluor 488 or 546 (Invitrogen, USA) was used to visualize the fluorescent signals. The nuclei were visualized with DAPI. The immunofluorescence signals were observed under a Zeiss Axioskop 2 fluorescence microscope, which was equipped with CCD camera and operated by MetaMorph 7.4 software. The images were acquired using the filter sets at Ex/Em of 490/520 nm for Alexa Fluor 488, Ex/Em of 550/575 nm for Alexa Fluor 546, and Ex/Em of 365/480 nm for DAPI-labeled nuclei.

PMNs counting. The number of PMNs from each brain section at the needle insertion site is calculated. PMN cell counting in mouse peripheral blood was performed with NDS solutions on slides after immersion in 95% methanol containing 5% acetic acid. A standard PMN antibody (ELANE, ab53457, Abcam). The digitized images (4 × 4 images under 20x lens) were acquired and stitched and the number of ELANE+ -neutrophils was automatically counted with CellSens software (Olympus).

NDS measurement. All behavioral tests in mice and rats were conducted in a quiet and low-lit room by an experimenter blinded with respect to the treatment groups. Pre-tests were done to exclude abnormal animals. Only animals with less than 20% foot-faults and animals that had no preference in forelimb placing were subjected to ICH. An individual test score and a combination test score from a battery of behavioral tests (Foot-fault, Forelimb Posturing, Postural Flexing, Wire and Circling) was used to measure the NDS, as we reported earlier45. 60.25. Grand composite NDS combined all the tests assumed an equal weight of each of the tests.

IL-27/anti-IL-27 Ab and LTF administration. Recombinant mouse IL-27 (rL-27, 577404, Biolegend, a heterodimer of mouse recombiant IL-27 EB13 and p28 linked by a GGSGGSGGGGTGGGS linker) at 50 ng/kg was administered i.v. at 0.5, 24, and 48 h after ICH to neutralize endogenous IL-27, as measured with rat anti-IL-27 Ab and LTF administration. We measured the LTF content in mature blood PMNs and expression of selected genes in BM-PMNs at 48 h with LTF ELISA, the LTF content in mature blood PMNs at 72 h with flow cytometry, and the NDS at 72 h and 7 days. For in vivo LTF experiments, rLTF (gift from PharmaReview Corporation, Houston, TX) was dissolved in saline and administered i.v. at 5 mg/kg, starting 30 min after ICH, plus 2.5 mg/kg, p.o. on days 2–7. Saline was used as the vehicle control. For the sub-acute study, recombinant mouse LTF (gift from PharmaReview Corporation, Houston, TX) was dissolved in saline and administered i.v. at 5 mg/kg, starting 30 min after ICH, plus 2.5 mg/kg, p.o. on days 1–7. Thereafter, we performed statistical analyses using the GraphPad and InStat programs and SAS 9.4 (Cary, NC). One-way analysis of variance (ANOVA) follow- ed by Newman–Keuls post-test was used for multiple comparisons. Paired t-test was used when two groups were compared. Two-way ANOVA followed by Bonferroni post-test to compare replicate means was used to compare NDS between two groups at different time points. Also, the longitudinal data analysis was performed using the mixed effects model.

Data availability. The data that support the findings of this study are available from the corresponding author on reasonable request.

Received: 30 December 2016 Accepted: 26 July 2017
Published online: 19 September 2017

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Acknowledgements

This work was supported by National Institute of Neurological Diseases and Stroke (NINDS), grants 5R01NS096308 and 2R44NS090650.

Author contributions

X.Z. contributed to the design of the study, carried biochemical characterization of neutrophil protein in vitro and in vivo, and assisted in analysis of behavioral and biochemical data and writing the manuscript. C.-H.L. conducted initial studies with the cytoprotective effect of IL-27 in vitro. G.S. performed all the animal surgeries and treatments. M.K. provided human lactoferrin and advised on all the experimental design aspects involving lactoferrin. S.-M.T. performed behavioral analyses. M.R.-O. conducted flow cytometry and assisted in writing the manuscript. J.A. initiated the project, conducted data analyses and provided overall guidance, and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00770-7.

Competing interests: Dr Kruzel is employed by PharmaReview Corporation, Houston, TX. The remaining authors declare no competing financial interests.
