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Clearance of neutrophils from ICH-affected brain by macrophages is beneficial and is assisted by lactoferrin and CD91

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Abstract

BACKGROUND: Within hours after intracerebral hemorrhage (**ICH**) onset, masses of polymorphonuclear neutrophils (**PMNs**) infiltrate the ICH-affected brain. After degranulation involving controlled release of many toxic antimicrobial molecules, the PMNs undergo rapid apoptosis, and then are removed by phagocytic microglia/macrophages (**MΦ**) through a process called efferocytosis. Effective removal of PMNs may limit secondary brain damage and inflammation; however, the molecular mechanisms governing these cleanup activities are not well understood. We propose that scavenger receptor CD91 on myeloid phagocytes especially in presence of CD91 ligand, lactoferrin (LTF, protein abundant in PMNs), plays important role in clearance of dead apoptotic PMNs (**ANs**).

METHODS: Mice/rats were subjected to autologous blood injection model of ICH. Primary cultured microglia were used to assess phagocytosis of ANs. Immunohistochemistry was employed to assess CD91 expression and PMNs infiltration. CD91 knockout mice selectively in myeloid phagocytes (Mac-CD91-KO) were used to establish the CD91/LTF function in phagocytosis and in reducing ICH-induced injury, as assessed using behavioral tests, hematoma resolution and oxidative stress.

RESULTS: Masses of PMNs are found in ICH-affected brain and they contain LTF. MΦ at the outer boarder of hematoma are densely packed, expressing CD91 and phagocytosing ANs.

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Our study indicates that CD91 scavenger receptor on microglia/macrophages uses lactoferrin for more effective removal of neutrophils from hemorrhage-affected brain to help limit neurological injury.

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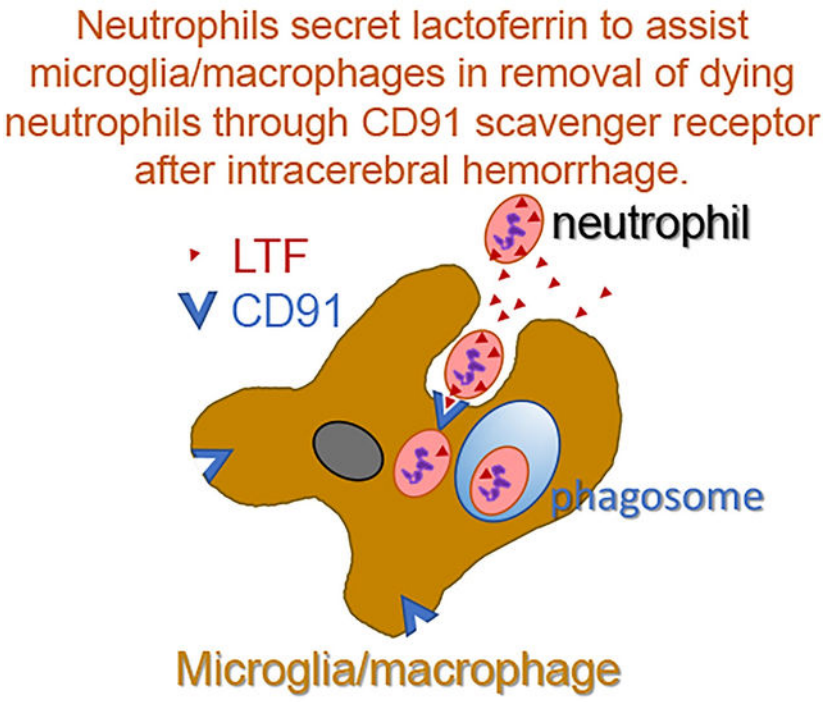
Microglia deficient in CD91 demonstrate defective phagocytosis of ANs, and mice deficient in CD91 (Mac-CD91-KO) subjected to ICH-injury have increased neurological dysfunction that is associated with impaired hematoma resolution (hemoglobin and iron clearance) and elevated oxidative stress. LTF that normally ameliorates ICH-injury in CD91-proficient control mice shows reduced therapeutic effect in Mac-CD91-KO mice.

CONCLUSIONS: Our study suggests that CD91 play a beneficial role in improving ANs phagocytosis and ultimately post-ICH outcome and that the beneficial effect of LTF in ICH is in part dependent on presence of CD91 on MΦ.

Graphical Abstract



Keywords

LRP1/CD91; neutrophil/PMNs; microglia; efferocytosis; lactoferrin; hematoma

INTRODUCTION

Intracerebral hemorrhage (**ICH**) is a devastating disorder with high mortality and poor prognosis, for which no drug therapy exists[1,2]. After ICH, the local microglia sense the damage and respond with the release of cytokines/chemokines[3,4], which orchestrate recruitment of **PMNs** and monocytes to the hematoma area, which may promote secondary brain injury[1,5].

The PMNs are the most abundant leukocytes in humans (~10^{11} are produced daily), which account for ~50–70% of all leukocytes[6]. After ICH, masses of PMNs infiltrate the ICH-affected brain[7]. Shortly after entering the brain parenchyma, the PMNs unleash an

arsenal of various constituents normally used for host defense, including reactive oxygen species (**ROS**) and proteases with potential to inflict tissue damage[8,9]. Normally, after degranulation, the PMNs undergo apoptosis *in situ* due to their inherent short life (half-life of only a few hours in circulation; and 1~2 days in tissue)[10,11] and are then removed by phagocytic microglia/macrophages (**MΦ**)[12] through a process called efferocytosis. This leads not only to the removal of the inflammatory cells themselves, but may also generate anti-inflammatory signal for MΦ to downregulate the ongoing inflammatory responses[13,14]. However, only limited studies explored the implications of clearance of the **ANs** to ICH pathogenesis.

MΦ recognize and phagocytose apoptotic but not freshly isolated neutrophils[15], indicating that efferocytosis is determined in part by signals provided by the ANs' surface molecules ("eat-me" signal)[16]. MΦ recognize these "eat me" signals via specific scavenger receptors, which bind to the ANs directly or indirectly via a variety of opsonizing proteins (so-called bridge molecules) that couple them together in a "ligand-receptor" manner. The best-characterized "eat-me" signal is the exposure of phosphatidylserine (**PS**) on the ANs plasma membrane[17,18]. Although there are no specific scavenger receptors for ANs, it has been suggested that CD91[19] contributes to the removal of ANs. CD91 is a heme scavenger receptor[20,21] involved in clearance of toxic heme (as in complex with hemopexin), dead cells and cellular debris and by that mean it plays an anti-inflammatory role[22]. Recently, protein lactoferrin (**LTF**), a highly abundant constituent of the PMNs' specific granules,[23] was suggested to represent a CD91 receptor ligand[24], which as such upon binding to CD91 may act as a bridging molecule to modify recognition/efferocytosis of apoptotic cells by MΦ.

Structurally, LTF is a single-chain iron binding glycoprotein that tightly binds/sequestrates ferric irons with uniquely high affinity of KD ~10^{-20}M[25]. Our recent studies suggest that PMNs upon infiltration into the ICH-affected brain release LTF, where the LTF by sequestrating iron can neutralize heme/iron' pro-oxidative activities[4]. LTF is a highly positively charged protein with an isoelectric point 8.7[26,27]. This likely renders LTF to be capable of coating/opsonizing apoptotic cells, by binding to the negatively charged surface molecules (e.g. phospholipids)[28,29]. Thus, LTF released by ANs may act as a bridge molecule, coupling the ANs with CD91 scavenger receptor, enhancing the recognition and efferocytosis of ANs by MΦ.

Here we explored a novel interaction between LTF and CD91 on MΦ as mechanism modulating clearance of AN and recovery after ICH using cell culture system and clinically-relevant rodent ICH mode.

MATERIAL AND METHODS

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Methodological details beyond description below are provided in the Supplemental Material. All animal studies followed the guidelines outlined in *Guide for the Care and Use of Laboratory Animals* from the NIH and were approved by the local Animal Welfare Committee and followed ARRIVE guidelines for rigorous reserach[30].

Mac-CD91-KO Mice.

To study CD91 in myeloid phagocytes, we generated microglia/macrophages-specific CD91-knockout (Mac-CD91-KO) mice by crossing LyzM-Cre[31] and CD91^{loxP}[32] mice. All the experimental groups of mice were age- and sex-matched.

Molecular Signaling and Genotyping.

PCR primers were used to detect CD91 recombination using a primer set (rec1, rec2, nonrec1 and nonrec2)(primers are listed in Table-S1).

ICH model.

ICH was induced by intra-striatal injection of autologous blood, as described[4,33]. Briefly, mice (25–30g) or Sprague-Dawley rats (250–300g) under anesthesia received intra-striatal blood infusion (5μl/5min for mouse, 35μl/7min for rat) through a 1-mm-diameter skull burr hole.

LTF administration.

The recombinant mouse LTF (mrLTF; Pharma-Review-Corporation) was injected i.v. at 10mg/kg in 150μl of saline (or saline alone/control), starting 3h after ICH, plus 5 mg/kg, i.p., daily on d1-d6 after ICH. For the microglia polarization study *in vitro*, the recombinant human LTF (rhLTF; Pharma-Review-Corporation, 50 μg/ml) was added to rat primary microglia at 16h or 0h prior to phagocytosis assay.

Animal perfusion and tissue collection.

Animals were intracardially perfused with saline. For histology/biochemical analyses, the whole brains or the striatum (area directly affected by ICH) were frozen then stored in −80°C prior to cryosectioning or RNA isolation.

Neurological/functional deficits (NDS).

All behavioral tests were conducted by an experimenter blinded with respect to the treatment assignment. The grand NDS is a composite score from a battery of behavioral tests (Footfault, Forelimb Placing, Postural Flexing, Wire, and Corner test), as we reported[4,33].

Hematoma size measurement.

Hematoma resolution was measured by residual hemoglobin (**Hb**) remaining in the hematoma-affected brain on d7 after ICH, as detailed[33].

Nonheme Iron Determination.

To localize iron in the brain sections, we used enhanced Perl's reaction[34].

Oxidative stress measurement.

Oxidative stress was determined by measuring the lipid peroxidation using 4-HNE (4-hydroxynonenal)[35].

PMN counting in brain after ICH.

Coronal sections representing the center of hematoma from mice at d1 and d7 after ICH were stained with anti-Neutrophil antibody (Abcam). To avoid sampling bias, we used MetaMorph 6.2 and image-stitching to count all the PMNs in ICH-affected hemisphere.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).

The RNA samples from brain tissue or cultured microglia were processed as described previously[4]. Primers are listed in Table-S1. The results were calculated as percentage change vs. the control.

Primary brain glia culture and microglia isolation.

The cortices of 1–2d-old postnatal rat and mouse pups were employed as source of microglia, as we described[4].

Blood PMNs isolation.

Peripheral rat blood was drawn through heart puncture from either the mother rat or the littermates (~20d-old) of the pups otherwise used for microglia cultures. The blood PMNs were purified with Ficoll-Paque gradient centrifugation[36]

Phagocytosis assay for apoptotic ANs in vitro.

The isolated PMNs were incubated at 45°C for 2h and then in a CO_2 cell culture incubator for 16h, followed by a 30min incubation in PKH26 (red fluorescence dye; Sigma-Aldrich) for visualization of ANs engulfment by the microglia.

Glycine and lysine.

Lysine or glycine was dissolved in DMEM+2% Fetal-Bovine-Serum to generate 0–5000μM solutions; and 50μg/ml of rhLTF was added into each tube. We added PKH26-labeled ANs into each of these tube at 2×10^6 ANs/ml and incubated shortly before applying the mixture onto the cultured microglia. The phagocytosis index was established 2h later.

Immunofluorescence.

Immunohistochemistry was performed as we described[4,7,33]. All antibodies are listed in Table-S2. The nuclei were visualized with DAPI.

Statistical Analyses.

Data are expressed as mean±SEM. For *in vitro* experiments, we pooled samples from three culture wells and repeated the experiments three times. We performed statistical analyses using the GraphPad, InStat and SAS programs. One-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test was used for multiple comparisons. Non-paired t-test was used when two groups were compared. PROC GENMOD procedure with PRAMA=GLM was used for the temporal changes of PMNs in the brain.

RESULTS

CD91 positive microglia/macrophage efferocytose ANs in ICH-affected brain.

Our earlier work and studies of others indicate that ICH elicits a quick and robust recruitment of blood PMNs into the hematoma-affected brain[7]. Here, using ICH in rats, we demonstrate that PMNs (the MPO^+ cells; we earlier validated that in the ICH-affected brain the MPO antibody labels only PMNs[7]) are found throughout the ICH-affected brain areas, including under corpus callosum where blood occasionally could be found in this ICH model (Fig-1). The PMNs in the ICH-affected brain contain high levels of LTF (Fig 2A–C), which could be seen within PMNs as intracellular puncta depicting specific granules (Fig-2C).

Along with infiltration of PMNs, microglia and macrophages (MΦ) also accumulate in the ICH-affected brain tissue. Using CD68 (Fig-1) to visualize the MΦ, a large number of MΦ was detected in the ICH-affected brain, primarily at the outer borders of the hematomas at 3 days after ICH. These MΦ enclosed not only hematoma but also hematoma-associated PMNs, forming a strategic zone for hematoma clearance and ANs efferocytosis.

Although CD91 is localized on many cell types, it is uniquely abundant on phagocytic cells such as microglia[37], monocytes and macrophages, where it acts as a multifunctional scavenger receptor assisting in the cleanup process[38,39]. Here we established that after ICH the perihematomal $CD11b^+$-MΦ express high levels of CD91 (Fig-2D&E). $CD91^+$-MΦ are almost exclusively abundant at the outer border of the hematomas, the location that is strategic for the clearance of hematoma. Using RT-PCR, we measured the temporal changes in CD91 mRNA in the ICH-affected brain tissues over 2 weeks post-ICH. The CD91 mRNA expression was modestly reduced within first hours after the ICH, but subsequently robustly increased starting from d1, peaked by d3–7 with 4–5 folds increase and still remained elevated by 2 weeks (Fig-2F).

To clarify if these $CD91^+$ MΦ participate in efferocytosis of ANs, we performed a double immunofluorescence using RP-1 (a specific marker for rat PMN) and CD91 antibodies in the ICH-affected brain tissue, 7d after ICH (Fig-3). Using this approach, we clearly detected the presence of $RP\text{-}1^+$-PMNs within the $CD91^+$-MΦ, suggesting that $CD91^+$-MΦ could indeed engulf the ANs in the ICH-affected brains.

LTF is important for efferocytosis of ANs by microglia through CD91, in vitro

To further clarify CD91 expression by microglia, we isolated microglia from the rat brain primary co-cultures. Using immunofluorescence, we established that these microglia strongly express CD91 (Fig-4A), and that they can effectively phagocytose ANs (Fig-4B). It is known that CD91 binds LTF[40], and LTF is produced by PMNs[23]. Thus, to explore if LTF may contribute to the phagocytic function of these $CD91^+$ microglia toward ANs, we either pre-incubated the microglia with rhLTF for 16h prior to exposing them to ANs (pre-LTF), or just added rhLTF to microglia only during ANs phagocytosis process (no pre-incubation). We found that under both treatment conditions rhLTF significantly improved the efferocytic function of microglia, with microglia pre-incubated with rhLTF demonstrating much stronger phagocytic enhancement (Fig-4B). These results suggest that pre-incubation with

LTF may modify these microglia' phenotype. Thus, to understand this process better, we interrogated the mRNA expression upon exposing the microglia to ANs with or without LTF. Exposure of microglia to ANs triggered upregulation of pro-inflammatory factors (including IL-1β, NOX1, TNFα), anti-inflammatory factors (Nrf2, TGFβ), and scavenger receptors (CD91 and CD36) (Fig-4D), suggesting a generation of a complex phenotype. Interestingly, inclusion of rhLTF significantly enhanced the production of anti-inflammatory factors (TGFβ, Arginase-1, but not Nrf2) and scavenger receptors (CD36 and CD91), while reduced pro-inflammatory responses (IL-1β, NOX1, TNFα) (Fig-4D). The transcriptomic changes induced by LTF could contribute to a shift of microglia signature toward M2-like phenotype with increased efferocytic activities. It is interesting to note that rhLTF alone (without ANs) imposed only subtle gene changes involving modest increased in the expression of CD36 (9.6%), CD91 (21.4%), TGFβ (15.1%), and Arg1 (10.7%), without significantly changing the expression of proinflammatory cytokines (IL-1β, TNFα).

The negative cell surface charges of ANs, in part driven by phosphatidylserine (PS) exposure on the outer plasma membrane, are the essential contributors to the efferocytosis process[17,18]. LTF by being highly positively charged could bind PS and in this capacity acts as a bridging molecule coupling ANs and CD91-expressing microglia. Thus, to assess if electrostatic charges of LTF could indeed contribute to enhanced coupling and thus efferocytosis, we preincubated the ANs with positive charged lysine (isoelectric point 9.59) to block interaction between opposite electric charges of LTF and ANs. We used neutrally charged glycine[41] (isoelectric point, 5.97) as control. We found that 5–5000 μM of glycine has no inhibitory effect on LTF-enhanced efferocytosis of ANs by microglia, while lysine at concentrations ≥500μM significantly reduced the abilities of LTF in stimulating efferocytosis (Fig-4C). This data suggests that charges-based attraction between ANs and LTF may in fact play a role in enhancing ANs efferocytosis by microglia, and that this mechanism could exist in addition to LTF-mediated efferocytosis enhancement that is regulated by changes in gene induction in MΦ.

CD91 deficiency weakens efferocytosis of ANs by microglia, in vitro.

First, to study the role of CD91 in mediating efferocytosis of ANs, we produced myeloid phagocyte-specific CD91 knockout mice (**Mac-CD91-KO**). From the postnatal pups of Mac-CD91-KO mice and $CD91^{LoxP}$ mice (control), we established brain glia cultures from which we purified microglia. Using RT-PCR, we confirmed the absence of CD91 mRNA in the microglia from Mac-CD91-KO mice (Fig-5A). Then, we subjected these microglia to phagocytosis assay and observed that CD91-KO microglia have significantly reduced (~33%) phagocytosis efficacy toward ANs, as compared to CD91 proficient microglia (Fig-5B), indicating that CD91 play important role in mediating engulfment of ANs.

Next, to clarify the contribution of LTF to CD91-mediated ANs efferocytosis, we included LTF in the phagocytosis assay. As anticipated, LTF significantly enhanced ANs efferocytosis in both CD91 deficient and proficient cells (Fig-5B), however, CD91 proficient microglia demonstrated 45.3% higher index of phagocytosis as compared to CD91 deficient cells, suggesting that CD91-LTF interaction is important in the ANs engulfment process.

The CD91 deficiency in myeloid phagocytes caused deficient cleanup and reduces the beneficial effect of LTF and lead to worse ICH outcome after ICH.

To better understand the role of CD91 on MΦ in ICH pathogenesis, we subjected Mac-CD91-KO and the control/CD91^{LoxP} mice to ICH and measured the neurological deficits and histological outcome. The Mac-CD91-KO mice appeared to be normal, including body weight, brain vasculature anatomy, or peripheral blood composition (data not included). However, in response to ICH, Mac-CD91-KO mice, as compared to control mice, suffered from more severe neurological deficits (NDS, Fig-6A) that coincided with defective hematoma clearance, as measured by residual levels of hemoglobin (Fig-6B) and iron (Fig-6C) in the hematoma-affected brain. Assessment of the brains for oxidative alteration with immunofluorescence for 4-HNE showed greater increase in lipid oxidation in Mac-CD91-KO vs. control mice (Fig-6D). Finally, we established that the number of PMNs remaining in the brain at d7 after ICH was higher in Mac-CD91-KO vs. control mice (Fig-6E). These overall suggest that CD91 on phagocytes play beneficial role in clearing hematoma and AN and protecting brain from damage caused by ICH.

Since CD91 on microglia binds LTF[24] and the CD91-LTF interaction may assist MΦ in recognition/engulfment of ANs, we tested if CD91 deficiency in MΦ could reduce the ability of LTF to benefit ICH-induced injury. Indeed, in contrast to CD91^{LoxP} mice that significantly benefited from rmLTF treatment, Mac-CD91-KO mice receiving rmLTF showed only a trend (no statistical difference) toward benefiting from LTF regarding neurological improvement (NDS), hematoma resolution (Hb residue), iron clearance, and oxidative stress (4-HNE) (Fig-6A–D). Also, whereas PMNs presence in the ICH-injured brain was initially modestly reduced by rmLTF, equally in CD91^{LoxP} and Mac-CD91-KO mice (likely due to LTF's anti-inflammatory effect) the number of brain PMNs on d7 after ICH was much lower in control mice than in Mac-CD91-KO (15% vs. 54% of d1' PMNs) (Fig-6E). These suggest that CD91 on MΦ is important in mitigating ICH-mediated damage, and that LTF could facilitate CD91-mediated cleanup, including efferocytosis of ANs that is beneficial for post-ICH recovery.

DISCUSSION

Following ICH, masses of PMNs infiltrate into the brain where it accumulates around the hematoma-affected tissue. This infiltration initiates within hours, peaks around 1–2d and then gradually resolves within 1–2 weeks[7]. PMNs are extremely short-lived and upon infiltration to the inflamed tissue, they shortly (hours to days) die through apoptosis and need to be fast removed by phagocytes to avoid secondary necrosis, lysis, spillage of a toxic moieties that further propagate pro-inflammatory responses and cause direct toxicity to the surrounding tissue[42].

Here, we demonstrate that MΦ after ICH can phagocytose dead PMNs, both in ICH-affected rodent brain and in cell culture system, and that scavenger receptor CD91 normally expressed on MΦ contributes to this engulfment process. Furthermore, we demonstrate that CD91 ligand lactoferrin (LTF)[24,43], a secretory protein highly abundant in the PMNs' specific granules[44] and delivered to the inflamed brain by these cells[4,45], acts as facilitator of the ANs engulfment. Ultimately, by using knockout animals that are selectively deficient

in CD91 in myeloid phagocytes, we showed that presence of CD91 on MΦ and availability of LTF together play beneficial roles in reducing neurological damage in a mouse model of ICH. These functional benefits were positively associated with improved brain hematoma resolution, iron removal from the ICH-affected brain, and reduced peri-hematoma oxidative insult.

MΦ recognize and phagocytose apoptotic, but not freshly isolated PMNs[15], suggesting that phagocytosis of PMNs is regulated by signaling molecules displayed on the ANs surface ("eat-me" signal)[16]. Apoptotic cells are normally negatively charged, in part due to externalized phosphatidylserine (PS) on their surface[46]. In contrast, LTF is highly positively charged (isoelectric point 8.7[26,27]) and therefore it demonstrates high attraction toward negatively charged molecules[47]. Since CD91 on MΦ acts as LTF receptor to which LTF can bind[24], we propose that LTF may function as a bridge between ANs and MΦ to facilitate more effective efferocytosis. In support of this model, we are showing here that LTF losses some of its efficacy to promote phagocytosis of ANs in presence of amino acid lysine, which is similar to LTF regarding carrying positive charges that can electrostatically outcompete charges-based interaction between LTF to AN. The equimolar to lysine concentration of glycine, a neutrally charged molecule[41], does not interfere with the ability of LTF to stimulate phagocytosis of ANs.

Applied acutely (only during the engulfment assay), LTF may indeed help in engulfment of ANs through charge-related modification (as suggested directly above). However, exposing microglia to LTF for hours prior to phagocytosis assay leads to further improvement in phagocytosis efficacy, suggesting that prolonged exposure to LTF may modify phagocytosis through distinct mechanisms. To understand this mechanism(s), we compared microglia gene expression changes triggered during engulfment of ANs with and without LTF treatment. Upon engulfment of ANs microglia underwent the complex transcriptional changes that involved induction of proinflammatory and anti-inflammatory responses, increased expression of scavenger receptor, and upregulation of anti-oxidative pathway, signified by the increased expression of Nrf2, a transcription factor and master regulator for anti-oxidative cell defense responses. Interestingly, this ANs-induced transcriptional changes were significantly modified in microglia that were pre-incubated with LTF, and resulted in a reversal of ANs-induced pro-inflammatory gene expression, further increased anti-inflammatory gene transcription, and further increase in scavenger receptor gene expression. These LTF-induced responses coinciding with the enhanced phagocytic responses suggest that LTF may change microglia phenotype to a more "healing" type - resembling M2 form.

While our findings here are important in implicating potential role of recombinant LTF as therapy for ICH as we suggested earlier [4,45,48], present work have additional pathophysiological relevance of the LTF delivered by inflammatory PMNs in the ICH-affected brain. Infiltration of PMNs to the hematoma site results in delivery of LTF to the site of ICH injury, mediating not only iron sequestration[4,45] but also promoting efficient removal of dead PMNs that entered brain after ICH, a sort of self-control for elimination. Normally, PMNs' primary function is to protect host organism from microbial invasion; therefore, their granules contain abundance of armories that upon uncontrolled PMNs lysis could produce direct injury to the hematoma surrounding viable brain and further augment

ICH-induced inflammation. Thus, we propose that LTF delivered by PMNs has dual beneficial function in ICH. One is to help sequestrate toxic iron derived from hemoglobin/heme and two is to help in removal of ANs by amplifying efficacy of CD91 scavenger receptor toward removal of ANs. Also, our ongoing studies suggest that in addition to removal of ANs the LTF can enhance removal of apoptotic neurons and the senescent RBCs, process that could be beneficial for post-ICH recovery.

Beside the “eat-me” signals on ANs such as phosphatidylserine, efferocytosis of ANs depends on presence of various factors in the microenvironment and the status of MΦ programming, which determines their readiness to engulf[49,50]. MΦs that are programmed toward so-called “alternative/healing” activation are usually more efficient at efferocytosis. Although, there are no specific set of the scavenger receptors for ANs, it has been suggested that CD36[51] and CD91[19] may play important role in the removal of ANs. In our ongoing studies, we indeed observed that CD36 neutralizing antibody *in vitro*, significantly reduced ANs efferocytosis by microglia by 16.8%, suggesting that CD36 plays some role in mediating efferocytosis of ANs (data not included). Here, we focused on CD91 (also referred to low density lipoprotein-related protein 1; LRP1), a well characterize heme scavenger receptor[20,21], which is expressed on macrophages where it assist in heme-hemopexin complex engulfment and RBC phagocytosis[52]. Now, we are showing that CD91 is abundantly expressed by cultured microglia and by phagocytes located by the site of hematoma after ICH. In fact, all the CD91-expressing $CD11b^{+}$-MΦ are selectively found in proximity to the hematoma and all of them displayed amoeba-like morphology, suggesting their pro-phagocytic phenotype[53]. In contrast, all the $CD11b^{+}$ MΦ located several cell layers away from the hematoma displaying ramified morphology and were $CD91^{-}$, suggesting that only $CD91^{+}$ MΦ for some reason have direct access to the hematoma site to engage in brain cleanup. This is likely functionally beneficial, as based on our finding $CD91^{+}$ microglia have enhanced phagocytic activity. It is also important to indicate that infiltration of PMNs to the ICH-affected brain starts within hours after ICH onset, resulting in masses of PMNs entering the hematoma[7]. Their location in the affected brain appears to be similar to the sites where activation of microglia and monocyte infiltration occurs. Thus, PMNs through degranulation-mediated LTF release, which occurs in response to ICH environment[45], could modify the adjacent $CD91^{+}$ MΦ by improving their phagocytic/cleanup functions.

LTF is an iron-binding glycoprotein that is synthetized during PMNs maturation in bone marrow, and is packed to so called specific granules of mature PMNs. It appears that among blood cells PMNs are the only cells synthetizing and carrying LTF. We have earlier demonstrated that LTF is neuroprotective against lysed RBC and iron induced cytotoxicity in primary rat neuron and also effective in reducing injury in rodents (mice and rats) models of ICH[4,45]. Thus, PMNs may work as a vehicle to carry and deliver LTF into the ICH-affected brain and provide therapeutic protection by sequestration of iron, modulation of MΦ gene expression and assisting in removal of apoptotic cells including ANs.

In conclusion our study suggests an important role of CD91 and LTF in promoting ANs clearance from the brain after ICH and implies that approaches targeting CD91 including through LTF may represent potential therapeutic value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms:

PMNs	polymorphonuclear neutrophils
ANs	apoptotic PMNs
MΦ	microglia/macrophages
PS	phosphatidylserine
LTF	lactoferrin
LyzM	lysozyme M
rhLTF	recombinant human LTF
rmLTF	recombinant mouse LTF

REFERENCES

1. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet neurology. 2012;11:720–731. doi: 10.1016/S1474-4422(12)70104-7 [PubMed: 22698888]
2. Qureshi AI, Mendelow AD, Hanley DF. Intracerebral haemorrhage. Lancet. 2009;373:1632–1644. doi: 10.1016/S0140-6736(09)60371-8 [PubMed: 19427958]
3. Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. The Journal of cell biology. 2010;189:1059–1070. doi: 10.1083/jcb.201004096 [PubMed: 20584912]
4. Zhao X, Ting SM, Liu CH, Sun G, Kruzel M, Roy-O'Reilly M, Aronowski J. Neutrophil polarization by IL-27 as a therapeutic target for intracerebral hemorrhage. Nature communications. 2017;8:602. doi: 10.1038/s41467-017-00770-7
5. Aronowski J, Hall CE. New horizons for primary intracerebral hemorrhage treatment: experience from preclinical studies. Neurological research. 2005;27:268–279. [PubMed: 15845210]
6. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest. 2000;80:617–653. [PubMed: 10830774]
7. Zhao X, Sun G, Zhang H, Ting SM, Song S, Gonzales N, Aronowski J. Polymorphonuclear neutrophil in brain parenchyma after experimental intracerebral hemorrhage. Translational stroke research. 2014;5:554–561. doi: 10.1007/s12975-014-0341-2 [PubMed: 24696130]
8. Sansing LH, Harris TH, Kasner SE, Hunter CA, Kariko K. Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. Acta neurochirurgica Supplement. 2011;111:173–178. doi: 10.1007/978-3-7091-0693-8_29 [PubMed: 21725751]
9. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. Stroke; a journal of cerebral circulation. 2011;42:1781–1786. doi: 10.1161/STROKEAHA.110.596718

10. Luo HR, Loison F. Constitutive neutrophil apoptosis: mechanisms and regulation. Am J Hematol. 2008;83:288–295. doi: 10.1002/ajh.21078 [PubMed: 17924549]
11. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nature reviews Immunology. 2002;2:965–975. doi: 10.1038/nri957
12. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity. 2005;22:285–294. doi: 10.1016/j.immuni.2005.01.011 [PubMed: 15780986]
13. Erwig LP, Henson PM. Clearance of apoptotic cells by phagocytes. Cell death and differentiation. 2008;15:243–250. doi: 10.1038/sj.cdd.4402184 [PubMed: 17571081]
14. Erwig LP, Henson PM. Immunological consequences of apoptotic cell phagocytosis. The American journal of pathology. 2007;171:2–8. doi: 10.2353/ajpath.2007.070135 [PubMed: 17591947]
15. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest. 1989;83:865–875. doi: 10.1172/JCI113970 [PubMed: 2921324]
16. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. Trends in immunology. 2011;32:350–357. doi: 10.1016/j.it.2011.04.009 [PubMed: 21782511]
17. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ. 1998;5:551–562. [PubMed: 10200509]
18. Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. Mol Cell. 2004;14:277–287. doi: S1097276504002370 [pii] [PubMed: 15125832]
19. Donnelly S, Roake W, Brown S, Young P, Naik H, Wordsworth P, Isenberg DA, Reid KB, Eggleton P. Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. Arthritis Rheum. 2006;54:1543–1556. doi: 10.1002/art.21783 [PubMed: 16645988]
20. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. Nature. 2001;409:198–201. [PubMed: 11196644]
21. Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ, Moestrup SK. Identification of the receptor scavenging hemopexin-heme complexes. Blood. 2005;106:2572–2579. doi: 10.1182/blood-2005-03-1185 [PubMed: 15947085]
22. Ascenzi P, Bocedi A, Visca P, Altruda F, Tolosano E, Beringhelli T, Fasano M. Hemoglobin and heme scavenging. IUBMB Life. 2005;57:749–759. [PubMed: 16511968]
23. Baggiolini M, De Duve C, Masson PL, Heremans JF. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. J Exp Med. 1970;131:559–570. [PubMed: 5413328]
24. Suzuki YA, Lopez V, Lonnerdal B. Mammalian lactoferrin receptors: structure and function. Cell Mol Life Sci. 2005;62:2560–2575. doi: 10.1007/s00018-005-5371-1 [PubMed: 16261254]
25. Lonnerdal B, Iyer S. Lactoferrin: molecular structure and biological function. Annual review of nutrition. 1995;15:93–110. doi: 10.1146/annurev.nu.15.070195.000521
26. Brock JH. The physiology of lactoferrin. Biochem Cell Biol. 2002;80:1–6.
27. Baker EN, Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. Cell Mol Life Sci. 2005;62:2531–2539. doi: 10.1007/s00018-005-5368-9 [PubMed: 16261257]
28. Boxer LA, Haak RA, Yang HH, Wolach JB, Whitcomb JA, Butterick CJ, Baehner RL. Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. J Clin Invest. 1982;70:1049–1057. [PubMed: 6290534]
29. Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, Rossi AG, Gregory CD. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. J Clin Invest. 2009;119:20–32. doi: 10.1172/JCI36226 [PubMed: 19033648]
30. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS biology. 2010;8:e1000412. doi: 10.1371/journal.pbio.1000412 [PubMed: 20613859]
31. Babaev VR, Yancey PG, Ryzhov SV, Kon V, Breyer MD, Magnuson MA, Fazio S, Linton MF. Conditional knockout of macrophage PPARgamma increases atherosclerosis in C57BL/6 and

low-density lipoprotein receptor-deficient mice. Arteriosclerosis, thrombosis, and vascular biology. 2005;25:1647–1653. [PubMed: 15947238]

32. Rohlmann A, Gotthardt M, Hammer RE, Herz J. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. J Clin Invest. 1998;101:689–695. [PubMed: 9449704]
33. Zhao X, Sun G, Zhang J, Strong R, Song W, Gonzales N, Grotta JC, Aronowski J. Hematoma resolution as a target for intracerebral hemorrhage treatment: role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. Annals of neurology. 2007;61:352–362. doi: 10.1002/ana.21097 [PubMed: 17457822]
34. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke; a journal of cerebral circulation. 2003;34:2964–2969.
35. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, Grotta JC, Aronowski J. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke; a journal of cerebral circulation. 2007;38:3280–3286. doi: 10.1161/STROKEAHA.107.486506
36. Oh H, Siano B, Diamond S. Neutrophil isolation protocol. J Vis Exp. 2008. doi: 10.3791/745
37. Marzolo MP, von Bernhardi R, Bu G, Inestrosa NC. Expression of alpha(2)-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. J Neurosci Res. 2000;60:401–411. [PubMed: 10797543]
38. LaMarre J, Wolf BB, Kittler EL, Quesenberry PJ, Gonias SL. Regulation of macrophage alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-gamma. J Clin Invest. 1993;91:1219–1224. [PubMed: 7680664]
39. Konishi Y, Okamura M, Konishi M, Negoro N, Yoshida T, Inoue T, Kanayama Y, Yoshikawa J. Enhanced gene expression of scavenger receptor in peripheral blood monocytes from patients on cuprophane haemodialysis. Nephrol Dial Transplant. 1997;12:1167–1172. [PubMed: 9198046]
40. Actor JK, Hwang SA, Kruzel ML. Lactoferrin as a natural immune modulator. Current pharmaceutical design. 2009;15:1956–1973. [PubMed: 19519436]
41. DD VANS. Physiology of the Amino Acids. Science. 1942;95:259–263. doi: 10.1126/science.95.2463.259 [PubMed: 17773703]
42. Greenlee-Wacker MC. Clearance of apoptotic neutrophils and resolution of inflammation. Immunol Rev. 2016;273:357–370. doi: 10.1111/imr.12453 [PubMed: 27558346]
43. Grey A, Banovic T, Zhu Q, Watson M, Callon K, Palmano K, Ross J, Naot D, Reid IR, Cornish J. The low-density lipoprotein receptor-related protein 1 is a mitogenic receptor for lactoferrin in osteoblastic cells. Molecular endocrinology. 2004;18:2268–2278. doi: 10.1210/me.2003-0456 [PubMed: 15178744]
44. Hansen NE, Malmquist J, Thorell J. Plasma myeloperoxidase and lactoferrin measured by radioimmunoassay: relations to neutrophil kinetics. Acta medica Scandinavica. 1975;198:437–443. [PubMed: 174393]
45. Zhao X, Ting SM, Sun G, Roy-O'Reilly M, Mobley AS, Bautista Garrido J, Zheng X, Obertas L, Jung JE, Kruzel M, et al. Beneficial Role of Neutrophils Through Function of Lactoferrin After Intracerebral Hemorrhage. Stroke; a journal of cerebral circulation. 2018;49:1241–1247. doi: 10.1161/STROKEAHA.117.020544
46. Kay JG, Grinstein S. Phosphatidylserine-mediated cellular signaling. Advances in experimental medicine and biology. 2013;991:177–193. doi: 10.1007/978-94-007-6331-9_10 [PubMed: 23775696]
47. Baker EN, Baker HM. A structural framework for understanding the multifunctional character of lactoferrin. Biochimie. 2009;91:3–10. doi: 10.1016/j.biochi.2008.05.006 [PubMed: 18541155]
48. Zhao X, Kruzel M, Ting SM, Sun G, Savitz SI, Aronowski J. Optimized lactoferrin as a highly promising treatment for intracerebral hemorrhage: Pre-clinical experience. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2021;41:53–66. doi: 10.1177/0271678X20925667 [PubMed: 32438861]
49. Mukundan L, Odegaard JI, Morel CR, Heredia JE, Mwangi JW, Ricardo-Gonzalez RR, Goh YP, Eagle AR, Dunn SE, Awakuni JU, et al. PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. Nature medicine. 2009;15:1266–1272. doi: 10.1038/nm.2048

50. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annual review of immunology. 2009;27:451–483. doi: 10.1146/annurev.immunol.021908.132532
51. Fadok VA, Warner ML, Bratton DL, Henson PM. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). Journal of immunology. 1998;161:6250–6257.
52. Patel M, Morrow J, Maxfield FR, Strickland DK, Greenberg S, Tabas I. The cytoplasmic domain of the low density lipoprotein (LDL) receptor-related protein, but not that of the LDL receptor, triggers phagocytosis. J Biol Chem. 2003;278:44799–44807. [PubMed: 12941948]
53. Vidal-Itriago A, Radford RAW, Aramideh JA, Maurel C, Scherer NM, Don EK, Lee A, Chung RS, Graeber MB, Morsch M. Microglia morphophysiological diversity and its implications for the CNS. Frontiers in immunology. 2022;13:997786. doi: 10.3389/fimmu.2022.997786 [PubMed: 36341385]
54. Savitz SI, Baron JC, Fisher M, Consortium SX. Stroke Treatment Academic Industry Roundtable X: Brain Cytoprotection Therapies in the Reperfusion Era. Stroke; a journal of cerebral circulation. 2019;50:1026–1031. doi: 10.1161/STROKEAHA.118.023927
55. Herz J, Clouthier DE, Hammer RE. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell. 1992;71:411–421. doi: 10.1016/0092-8674(92)90511-a [PubMed: 1423604]
56. Gautam A Classification of White Blood Cells Based on Morphological Features. IEEE Xplore Digital Libarory. 2014;978-1-4799-3080-7/14.
57. Zhao X, Strong R, Zhang J, Sun G, Tsien JZ, Cui Z, Grotta JC, Aronowski J. Neuronal PPARgamma deficiency increases susceptibility to brain damage after cerebral ischemia. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29:6186–6195. [PubMed: 19439596]
58. Asahi M, Asahi K, Wang X, Lo EH. Reduction of tissue plasminogen activator-induced hemorrhage and brain injury by free radical spin trapping after embolic focal cerebral ischemia in rats. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2000;20:452–457. doi: 10.1097/00004647-200003000-00002 [PubMed: 10724108]
59. Weinfeld A, Lundin P, Lundvall O. Significance for the diagnosis of iron overload of histochemical and chemical iron in the liver of control subjects. Journal of clinical pathology. 1968;21:35–40. [PubMed: 5697333]
60. Zhao X, Sun G, Zhang J, Ting SM, Gonzales N, Aronowski J. Dimethyl Fumarate Protects Brain From Damage Produced by Intracerebral Hemorrhage by Mechanism Involving Nrf2. Stroke. 2015. doi: 10.1161/STROKEAHA.115.009398
61. Zhao X, Wang H, Sun G, Zhang J, Edwards NJ, Aronowski J. Neuronal Interleukin-4 as a Modulator of Microglial Pathways and Ischemic Brain Damage. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2015;35:11281–11291. doi: 10.1523/JNEUROSCI.1685-15.2015 [PubMed: 26269636]

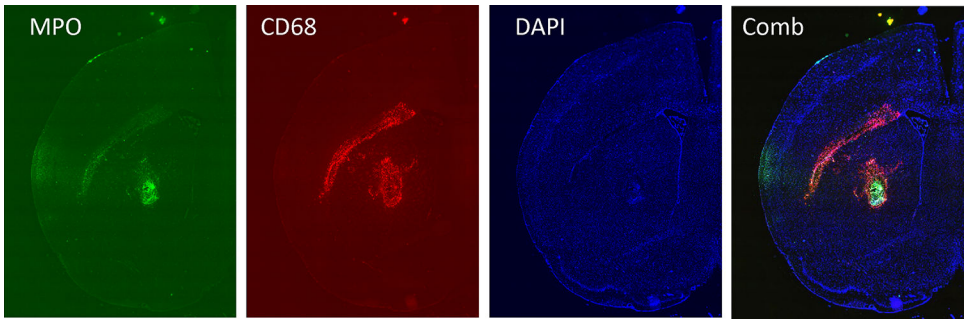

Fig 1.

Representative immunofluorescence to label PMNs (MPO^+/Green) and MΦ ($CD68^+$/red) showing distinct spatial distribution of PMNs and MΦ in the ICH-affected rat brain at 3d after ICH. The nuclei are labeled with DAPI/blue. A combination of MPO^++$CD68^+$/Comb staining is used to visualize co-distribution of PMNs and MΦ.

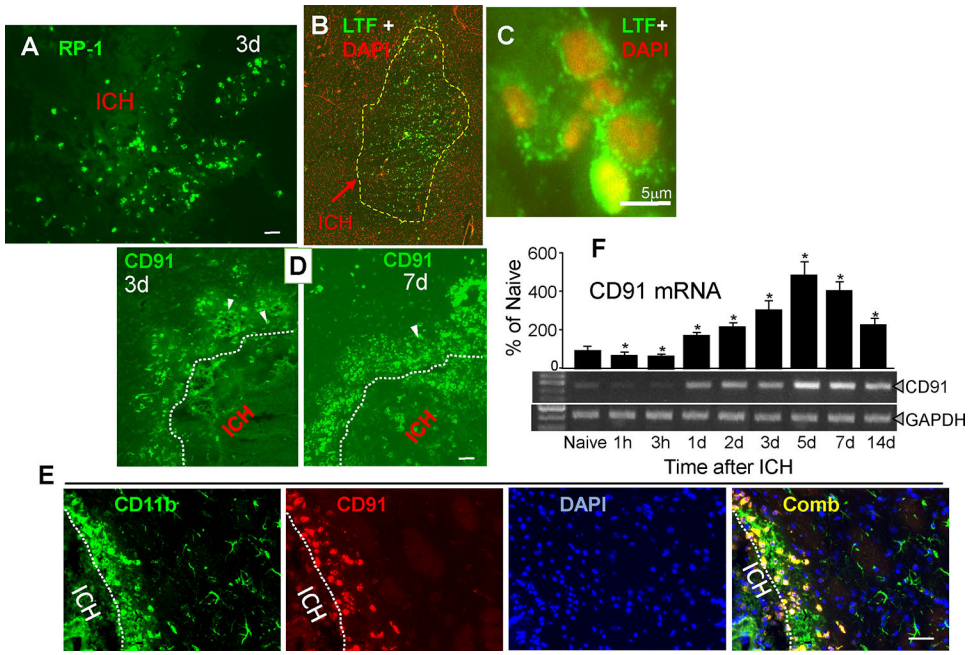

Fig 2.

(A) Immunofluorescence to localize PMNs (RP-1/green) showing distribution of PMNs in the ICH-affected rat brain at d3 after ICH. Scale bar=10μm. **(B&C)**Representative immunofluorescence of LTF/green in the ICH-affected brain. The LTF is localized intracellularly in PMNs (multi-lobular nuclei/red) found around the hematoma (dashed line) at d3 after ICH. **(D)**Immunofluorescence of CD91 in the ICH-affected brains at 3d and 7d after ICH. The arrowheads indicate the $CD91^+$-cells/green at the border of hematoma (doted lines). Scale bar=50μm. **(E)**Double immunofluorescence of $CD11b^+$/green and $CD91^+$/red to demonstrate the peri-hematoma localization of CD91 on MΦ at d3 after ICH. Note that all the $CD11b^+$-MΦ with the amoeba-like morphology are $CD91^+$ and are confined to the outer margin of hematoma. Scale bar=100μm. **(F)**Bar graph and RT-PCR gel image of CD91 mRNA expression levels in the ICH-affected subcortical striatum of Naïve animals and animals at 1h-14d after ICH. GAPDH acts as control. The data are mean±SEM (n=5). *P≤0.05, vs. Naïve group. Analyzed with One-Way ANOVA, followed by Newman-Keuls post-hoc test.

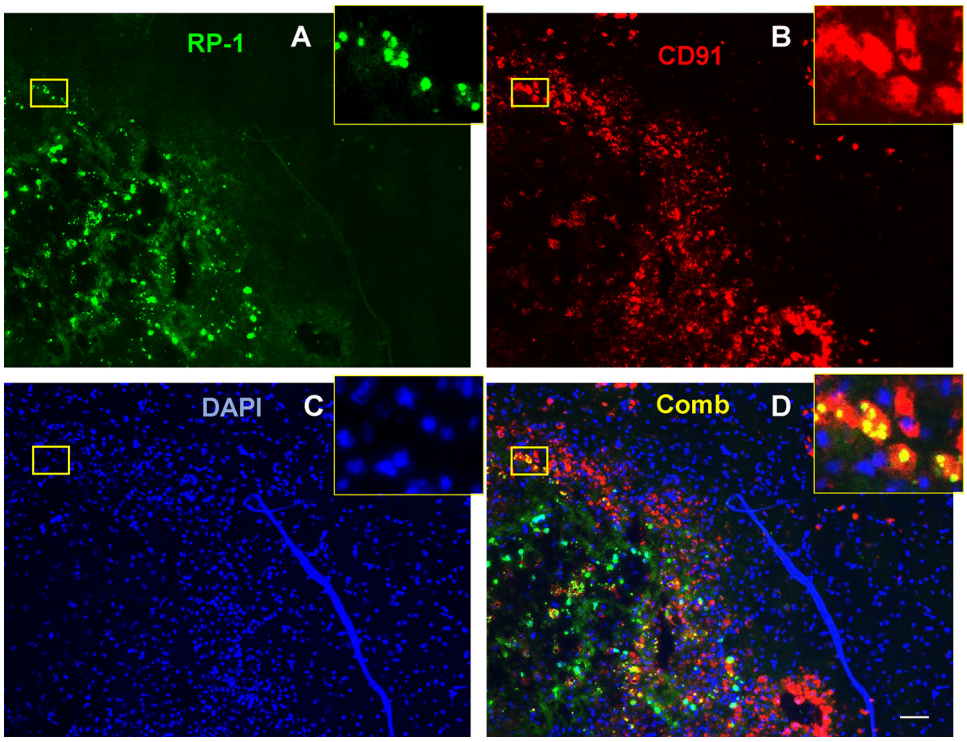

Fig 3.

Double immunofluorescence of RP-1/green and CD91/red in the ICH-affected rat brain at d3 after ICH. The nuclei are labeled with DAPI/blue. A highlighted window is showing the efferocytosed PMNs within the $CD91^{+}$-MΦ at the hematoma border zone. Scale bar=100μm.

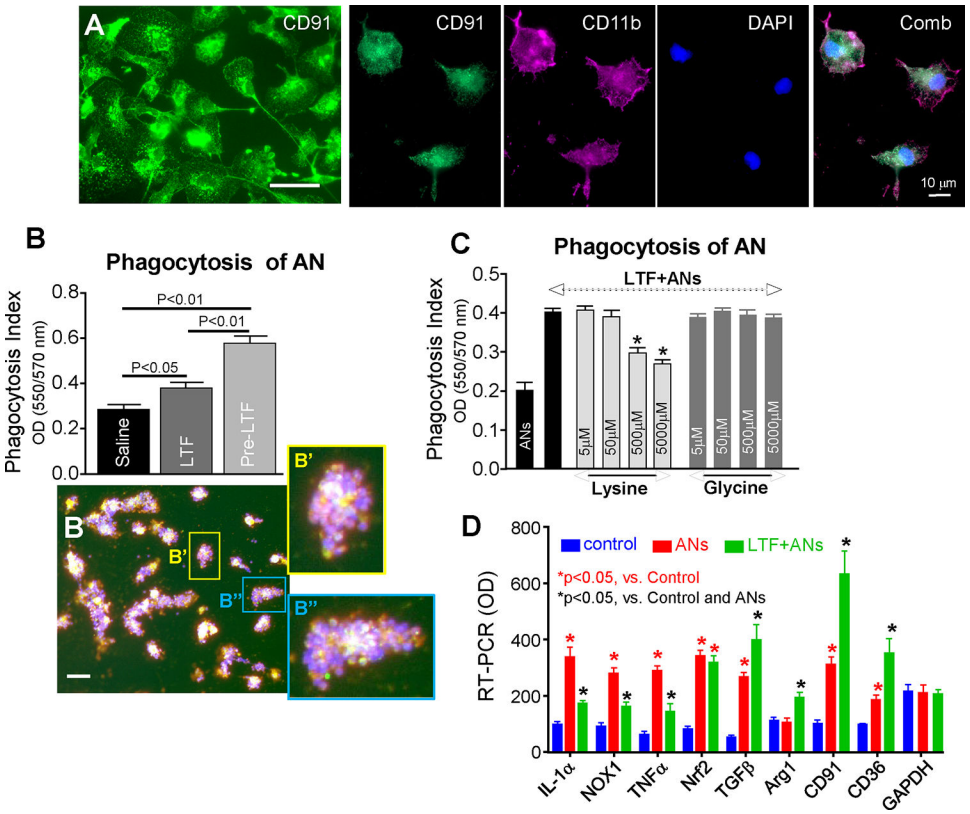

Fig 4.

(A) Double immunofluorescence of CD91 and CD11b in rat primary microglia showing that CD11b-labeled microglia express CD91. Scale bar=50μm. **(B)**Representative immunofluorescence of Iba-1^{+}-rat microglia/green at 2h after adding PKH26-labeled ANs/red (target of phagocytosis). The nuclei are labeled with DAPI/blue. Scale bar=50μm. **(B'&B"),** a highlighted MΦ containing engulfed ANs. A quantifying bar-graph showing that microglia treated with rhLTF at the time of adding ANs (LTF) or with 16h pre-treatment (Pre-LTF) promote ANs efferocytosis. The data are mean±SEM (n=3). **(C)**Phagocytosis Index upon exposing rat microglia to ANs with or without LTF in presence of 5–5000μM of lysine or glycine. The data are mean±SEM (n=3). *p≤0.05, vs. LTF alone. **(D)**Bargraph of RT-PCR quantitation showing mRNA changes upon exposing microglia to ANs with or without rhLTF. The rat microglia were incubated in saline or 50μg/ml rhLTF in presence of ANs for 16h. The mRNA changes were quantified by RT-PCR (n=3). The red asterisks indicate p≤0.05, vs. vehicle control and black asterisk indicates p≤0.05, vs. ANs alone and control. Analyzed with One-Way ANOVA, followed by Newman-Keuls post-hoc test.

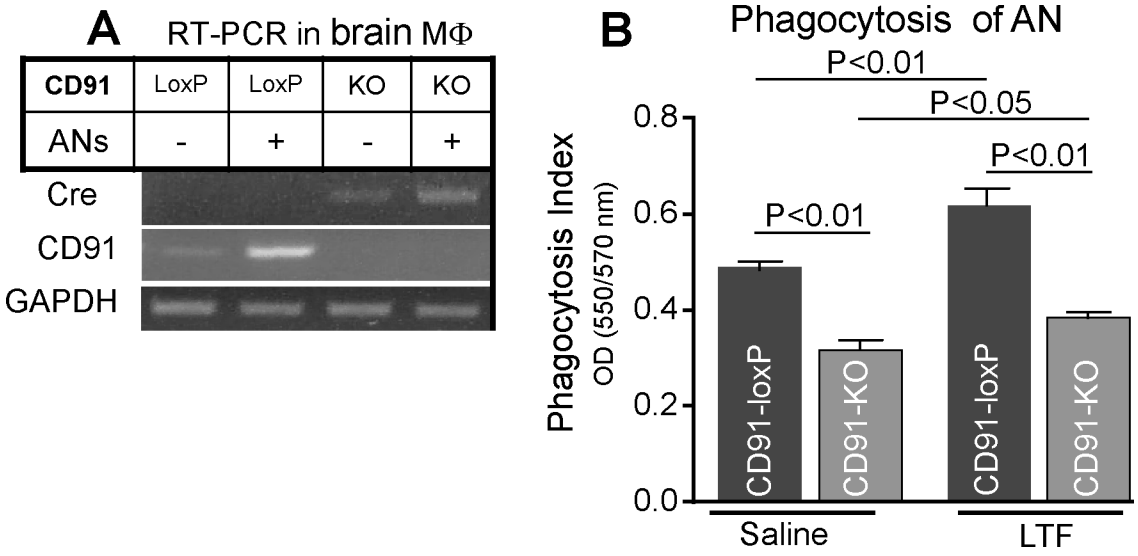

Fig 5.

(A) Microglia in culture were produced from CD91^{LoxP} (left 2 lanes) or Mac-CD91-KO (right 2 lines) mice. RT-PCR gels confirm Cre expression in microglia from the Mac-CD91-KO that coincides with loss of CD91 mRNA. GAPDH=internal control. **(B)**Phagocytosis Index of ANs engulfment by microglia from Mac-CD91-KO and CD91^{LoxP} mice with/without 50μg/ml of mrLTF. Data are mean±SEM (n=3). In CD91-KO microglia, the effects of LTF in promoting phagocytosis is significantly weaker than in the control microglia. Analyzed with One-Way ANOVA, followed by Newman-Keuls post-hoc test.

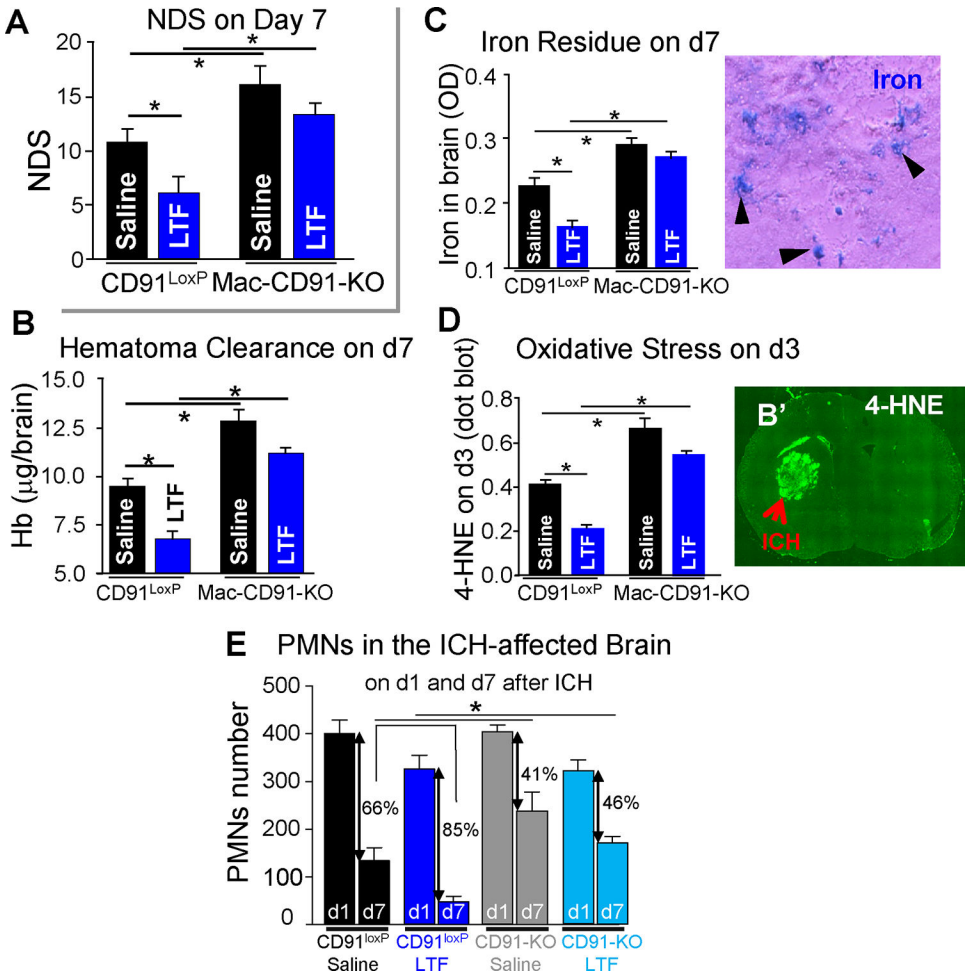

Fig 6. Outcome measurement of Mac-CD91-KO and the CD91^{LoxP} control mice after ICH.

(**A**)Neurological deficit score (NDS)(n=16) on d7, (**B**)Hematoma clearance (n=7) on day 7, (**C**)Oxidative stress measured on d3 (by immunofluorescence of 4-HNE)(n=5), (**D**)Iron clearance (n=7) on d7, and (**E**)PMNs count (n=5/group/timepoint) in the ICH-affected brain on d1 and d7 after ICH in Mac-CD91-KO and CD91^{LoxP} mice with/without mrLTF. The mrLTF was injected i.v. 3h after ICH at 10mg/kg in 150μl of saline (vs. saline control), plus 5mg/kg, i.p., daily on d1-d6 after ICH. The data are mean±SEM. *p<0.05. Analysis with One-Way ANOVA, followed by Newman-Keuls post-hoc test in A-D, and with PROC GENMOD procedure with PRAMA=GLM in E for the timely change of PMNs in the brain.