Microglia are important immune cells in the central nervous system. Replacement of mutated microglia by wild-type cells through microglia replacement by bone marrow transplantation can correct gene deficiencies. However, the limited availability of bone marrow cells may restrict its potential of becoming a widely used clinical treatment. Here, we introduce a potentially clinical feasible strategy achieving efficient microglia replacement by peripheral blood (Mr PB) cells in mice, boosting the donor cell availability. We named it Mr PB.
Protocol

Protocol for microglia replacement by peripheral blood (Mr PB)

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SUMMARY

Microglia are important immune cells in the central nervous system. Replacement of mutated microglia by wild-type cells through microglia replacement by bone marrow transplantation can correct gene deficiencies. However, the limited availability of bone marrow cells may restrict its potential of becoming a widely used clinical treatment. Here, we introduce a potentially clinical-feasible strategy achieving efficient microglia replacement by peripheral blood cells in mice, boosting the donor cell availability. We named it microglia replacement by peripheral blood (Mr PB).

For complete details on the use and execution of this protocol, please refer to Xu et al. (2020). The original abbreviation of this microglia replacement strategy is mrPB. We hereby change the name to Mr PB.

BEFORE YOU BEGIN

Prepare the PLX5622-formulated AIN-76A chow diet
Prepare the following recipe: 1.2 g (CAS Number 1303420-67-8) PLX5622 per kg AIN-76A diet. The PLX5622 was formulated by SYSY Bio. One adult mouse (25 g) consumes about 3.5 g chow diet per day.

Stored at −20°C until use. The PLX5622-formulated diet is valid for at least one year.

Prepare CX3CR1+/GFP mice
Cross CX3CR1+/GFP with C57BL/6J to obtain the CX3CR1+/GFP mice.

Prepare CX3CR1-CreER::Ai14 mice
Cross CX3CR1-CreER::Ai14 with Ai14 to obtain the CX3CR1+/CreER::Ai14wt/mut (CX3CR1-CreER::Ai14) mice.

KEY RESOURCES TABLE

| REAGENT OR RESOURCES | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | MedChemExpress (MCE) | HY-114153 |
| Tamoxifen citrate | Aladdin | T101373 |

(Continued on next page)
MATERIALS AND EQUIPMENT

Prepare the following solutions

- Tamoxifen stock solution (20 mg/mL) in corn oil. Store at –20°C for at most half a year.
- Neomycin containing acid water (1.1 g/L, adjust pH to 2–3 by HCl). Store at 4°C for at most one month.
- Ketamine (10 mg/mL) and xylazine (1 mg/mL) mixture in PBS. Store at –20°C for at most half a year.

  Note: Dilute the ketamine stock (50mg/mL) to 10mg/mL, and add xylazine to a concentration of 1mg/mL.

- Meloxicam working solution (0.1 mg/mL in PBS). Store at –20°C for at most half a year.

STEP-BY-STEP METHOD DETAILS

For clinical trials, Mr PB is conducted by exchange transfusion. For the lineage tracing for Mr PB by mouse models, it can be conducted by either CX3CR1-CreER::Ai14 mice or parabiosis. In this protocol, we will introduce these two lineage tracing approaches one by one.

Protocol I: Mr PB lineage tracing by CX3CR1-CreER::Ai14 mouse

The overall procedure of Mr PB by CX3CR1-CreER::Ai14 mouse is outlined in Figure 1.

Continued
Labeling the CNS endogenous microglia

**Timing:** 4 days

In this procedure, we utilize CX3CR1-CreER::Ai14 mice for lineage tracing. A subpopulation of monocytes also express CX3CR1 (Jung et al., 2000; Yona et al., 2013), which will be replaced by the differentiation of CX3CR1-negative cells. To specifically label the CNS endogenous microglia without affecting peripheral blood cells, we induce tdTomato expression in the CX3CR1-CreER::Ai14 neonatal mouse from P1 to P4 with tamoxifen administration. At 2-month-old, almost all endogenous microglia express tdTomato while the circulating blood cells are tdTomato-negative (Huang et al., 2018a; Huang et al., 2018b; Parkhurst et al., 2013; Xu et al., 2020).

**Note:** A period of at least 15- to 30-day is necessary to allow the tdTomato pre-labeled circulating monocytes to turnover.

1. Dilute tamoxifen stock solution of 20 mg/mL to working solution 2 mg/mL with corn oil.
2. Intragastrically inject 25 µL tamoxifen working solution each day from P1 to P4.
3. Clean the oil outflows of the pups with 75% ethanol. Mix the pups with feces from the parents before returning them to the home cage.

Microglial depletion

**Timing:** 14 days

This procedure achieves efficient microglial depletion (usually > 99% in the brain cortex and 100% in the retina) in the adult mouse CNS with the CSF1R inhibitor PLX5622 (Huang et al., 2018a; Huang et al., 2018b; Xu et al., 2020).

4. At 2-month-old, feed the tamoxifen-administered CX3CR1-CreER::Ai14 mouse with PLX5622-formulated chow diet from D0 to D14 ad libitum.
5. Replace and supply the PLX5622-formulated chow diet every 3 days.
6. Before the irradiation treatment, treat the mouse with neomycin containing acid water (1.1 g/L, adjust pH to 2–3 by HCl) ad libitum from D11. The acid water prevents most bacterial contaminations (Duran-Struuck and Dysko, 2009).

**Note:** According to our experience, at the specific pathogen free (SPF) animal facility, more than 95% of microglia in the brain cortex and 100% of retinal microglia in the outer plexiform layer (OPL) are able to be ablated at day 7 of PLX5622 administration. At day 14, more than 99% of brain microglia in the cortex and all retinal microglia are ablated (Huang et al., 2018a; Huang et al., 2018b). In contrast, microglial depletion efficiency will be dampened in a non-SFP animal facility. The efficiency of microglial depletion is critical for the replacement efficiency. We strongly recommend a 14-day microglial depletion.
△ CRITICAL: The animals should be kept at a specific pathogen-free (SPF) animal facility. Otherwise, the microglial depletion efficiency is relatively low in the "dirty environment", which will influence the replacement efficiency.

**Head irradiation to the CX3CR1-CreER::Ai14 mice**

- **Timing:** 4 hours

7. Change the PLX5622-formulated chow with sterilized AIN-76A control diet (CD) 2–3 h before the irradiation at D14.
8. Deeply anesthetize the CX3CR1-CreER::Ai14 mouse by the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) through the intraperitoneal injection.
9. Use soft lead piece (3 mm thickness) (Figure 2A) to cover the body of CX3CR1-CreER::Ai14 mice, only exposing the head of the mouse (from nose tip to the ear tips) (Figure 2B).

**Note:** The lead piece weight should not be imposed upon the mouse.

10. Set the X-ray irradiation dosage to 9 Gy on the irradiator (Radsource, RS-2000). Put the mouse cage into the irradiator and initiate irradiation.

△ CRITICAL: Please make sure the irradiation is X-ray. We found that the replacement efficiency by Gamma-ray is quite low. The typical irradiation time is around 7 min.

11. After the irradiation, remove the lead piece from the mice and put them to the home cage.
12. Feed the irradiated mouse with sterilized CD for recovery of 30 days ad libitum. Meanwhile, treat the mouse with neomycin containing acid water for the first 2–3 weeks after irradiation.

**Protocol II: Mr PB lineage tracing by parabiosis**

The overall procedure of Mr PB by parabiosis is outlined in Figure 3.
Parabiosis surgery

Timing: 14 days

The procedure is mostly following (Kamran et al., 2013) with minor changes. For those who wish to check the step-by-step details, we recommend to refer to the video of this paper.

13. To ensure the cohabitation of harmony, co-house a female C57BL/6J mouse with an age and body weight matched female CX3CR1+/GFP mouse for 2 weeks before the surgery. Typically, we utilized 1.5-month-old to 6-month-old mice for parabiosis experiment.

14. Flanks on opposite sides of two mice were shaved one day before the surgery.

15. Clean the operation bench and heating pad with 75% ethanol.

16. Cover the operation bench with a clean drape.

17. Sterilize the following surgical tools by a bead sterilizer for 20 s: toothed forceps, smooth forceps, ophthalmic scissors and needle holder. Put the tools on sterile paper towel. Wait for around 5 min till the tools cooling down before the experiment.

18. After deeply anesthetized by isoflurane or the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), flanks from the elbows to the knees on the opposite sides of the two mice were carefully cleaned by Betadine and ethanol solution, before the incisions are made. Put the mouse pair on a heating pad to maintain the body temperature.

19. Cut the skin at the knee joints of the mice. Insert the smooth forceps tips into the cut and detach the skin from the subcutaneous fascia from caudal to rostral until reaching the elbow joints with the help of tissue forceps. Cut the skin and expose approximately 1 cm tissues free of skin.

Note: Use the scissors tips to detach the skin by expanding the tips. Try to avoid bleeding as much as possible, especially when reaching the armpit of the mouse, by carefully enlarging the space between skin and subcutaneous fascia using the tip of scissors, and cut the skin with scissors thereafter. Keep the exposed tissue moistened with the sterile saline.

△ CRITICAL: Arrange the mouse pair on their unshaved side down and with their backs towards to each other.

20. Surgically connect the exposed elbow joints of two mice with non-absorbable 3-0 sutures. Lift and bend the elbow of the mouse on your right side. From right to left, pierce the suture needle through the muscle on the olecranon. Lift and bend the elbow of the mouse on your left side. Also, from right to left, pierce the suture needle through the muscle on the olecranon.

△ CRITICAL: Use the non-absorbable suture. We start from the mouse on the right side because most of the experimenters were right-handed.
21. Repeat step 20. With the suture thread tightly around the elbow and do not hurt the muscle. Firmly tighten a surgical knot.
22. Repeat step 20 to 21 for the knee joints.

△ CRITICAL: Pay attention not to rupture the major blood vessels near the femur.

23. Suture ventral skins of the two mice by 5-0 absorbable sutures from the elbow to the knee.

△ CRITICAL: To prevent from hurting the skin, areas around the elbows and the knees should be sutured tightly with double knots and using the interrupted stitch type.

24. Put the mouse pair stomach down and continue suture the dorsal ends with the double surgical knot. Verify the continuity of the suture and confirm the lack of openings.
25. Apply erythromycin ointment to sutured areas. Subcutaneously inject 0.5 mL sterilized normal saline to each mouse near the joint skin. Keep the mouse partners on the heating pad until they are recovered.
26. Put the mice (Huang et al., 2018b) back to a cage with clean monolithic bedding material (paper towel for example), and put enough sterile water-soaked wet chow on floor of the cage. To prevent bacterial infections, add neomycin in the drinking water (1.1 g/L). Return the cage to the animal facility.
27. Administer meloxicam (1 mg per kg body weight, Sigma) to relieve pain and limit potential infection for at least 48 h.
28. Keep supplying sterile chow on the floor of the cage for one week.
29. The mouse pair were able to feed and drink without difficulty by the time of 14 days post-surgery (D0-D14). One can easily verify the successful equilibration of the blood of the parabiotic WT and GFP positive mice by flow cytometric analysis (Huang et al., 2018b). After 14 days of recovery, the blood cells are thoroughly exchanged (~50% exchange rate).

Microglial depletion

⊗ Timing: 14 days

This procedure achieves efficient microglial depletion in the adult mouse CNS with the CSF1R inhibitor PLX5622 (Huang et al., 2018a; Huang et al., 2018b; Xu et al., 2020).

30. After 14 days post parabiosis surgery, feed the parabiotic mice with PLX5622-formulated chow diet at D14 to D28 ad libitum. Replace the PLX5622-formulated chow diet every 3 days.
31. Before the irradiation treatment, treat the mouse with neomycin containing acid water (1.1 g/L, pH 2 to 3) ad libitum from D25.

△ CRITICAL: The animals should be kept at a specific pathogen-free (SPF) animal facility. Otherwise, the microglial depletion efficiency is relatively low, which may influence the replacement efficiency.

X-ray irradiation to the parabiotic C57BL/6J mouse

⊗ Timing: 1 h

32. Change the PLX5622-formulated chow with sterilized AIN-76A control diet (CD) 2–3 h before the irradiation at Day 28.
33. Deeply anesthetize the parabiotic mice by the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg).
34. Use soft lead piece (3 mm) to cover the whole body of the parabiotic CX3CR1+/GFP mouse, while expose the parabiotic C57BL/6J mouse (Figure 4).

*Note:* The lead piece weight should not be imposed upon the mouse.

35. Set the X-ray irradiation dosage to 9 Gy on the irradiator (Radsource, RS-2000). Put the mouse cage into the irradiator and initiate irradiation.

△ CRITICAL: Please make sure the irradiation is X-ray. We found that the replacement efficiency by Gamma-ray is quite low.

36. After the irradiation, remove the lead piece and put the mouse to the home cage.

37. Feed the irradiated mouse with sterilized CD for recovery of 30–60 days *ad libitum.* Meanwhile, treat the mouse with neomycin containing acid water for the first 2–3 weeks after irradiation.

**EXPECTED OUTCOMES**

For CX3CR1-CreER::Ai14-based Mr PB strategy, the majority of IBA1-positive microglia-like cells in the brain are tdTomato-negative cells at D44 (typically > 80%) (Figure 5). Peripheral blood cell-derived microglia-like Mr PB cells are tdTomato-negative while the endogenous microglia are tdTomato-positive.

For parabiosis-based Mr PB strategy, around half of the IBA1-positive microglia-like cells in the brain of the parabiotic C57BL/6J mouse are GFP-positive at D88 (Figure 6). The GFP-positive microglia-like Mr PB cells in the CNS of the parabiotic C57BL/6J mouse are derived from the peripheral blood cells from the parabiotic CX3CR1+/GFP mouse.

**LIMITATIONS**

Though the parabiosis mode offered a novel and non-limiting type of donor cells to replace endogenous microglia, the way it supplements the donor is not ideal, because the two mice were joint together by surgical procedure. An ideal way to supplement the donor cell is to perform adoptive transfer of leukocytes, which applicable in the human case, because the blood source is not so...
restricted. But for the mice, to obtain constant supply of leukocytes, lots of mice have to sacrificed. Parabiosis is a method to avoid such massive killing of mice.

To achieve a microglia replacement efficiency of 99% during the 14-day treatment of PLX5622, an SPF environment is preferred. But a lot of facilities do not have such environment, the microglia depletion efficiency will be somewhat comprised. Also, the replacement condition method, i.e., irradiation, is somewhat picky about the source: The Gamma-ray source is inferior compared to the X-ray source.

Figure 5. In the CX3CR1-CreER::Ai14-based Mr PB strategy, the majority of IBA1-positive microglia-like cells in the brain are tdTomato-negative cells at D44
Green: IBA1; red: tdTomato; blue: DAPI.

Figure 6. In the parabiosis-based Mr PB strategy, around half of the IBA1-positive microglia-like cells in the brain of the parabiotic C57BL/6J mouse are GFP-positive
Green: GFP, red: IBA1, blue: DAPI.
The replacement efficiency of Mr PB is lower than that of Mr BMT, possibly because of the donor difference, for the bone marrow donor cells contain progenitors, which are highly proliferative compare to leukocyte in the Mr PB case. But still one merit of Mr PB over Mr BMT is that its donor source is relatively unlimited.

**TROUBLESHOOTING**

**Problem 1**
Low efficiency of microglia depletion (step 4)

**Potential solution**
In our experience, an SPF environment and chemically defined feeding chows are crucial for efficient depletion of microglia. Also, change the PLX5622 chow every 3 days in case the drug decomposes at the room temperature. Following these rules, the efficiency of microglia depletion can achieve as high as 99% after 14 days of PLX5622 treatment.

**Problem 2**
Mouse death after anesthetization during irradiation (step 8)

**Potential solution**
We strongly suggest that operator strictly follow our anesthetizing protocol, i.e., using ketamine (100 mg/kg) and xylazine (10 mg/kg) in I.P. injection manner. This is the safe procedure we tested. Also, never let the weight of soft lead piece squash the anesthetized mice, or it will cause breath difficulty to the mice.

**Problem 3**
Mouse death after parabiosis operation (step 29).

**Potential solution**
At the first few days post parabiosis operation, since the mice have difficulties in movement, put wetted chows on the cage floor to aid the mice’s feeding. Also, give mice analgesia injection (me-loxicam) twice a day subcutaneously for the first 48 h, not just for pain easing, but also for body fluid supplement. Supplement the drink water with neomycin to prevent infection.

**Problem 4**
Low microglia replacement efficiency (step 12 and 37)

**Potential solution**
First, check the microglia depletion efficiency, and ensure that 99% microglia were depleted before performing Mr PB. Second, in the head irradiation mode, make sure that the mouse head is exposed (from the ear tip to the nose). Third, ensure that the mice remain sedated duration irradiation, by strictly following our anesthetizing protocol. Third, please make sure that the irradiation source was X-rayed based. We found that Gamma-ray irradiation generated a low replacement efficiency.

**Problem 5**
Mice death after irradiation (step 37)

**Potential solution**
Since the irradiation is not lethal, usually this does not happen. But in the parabiosis mode, the death risk is still great since the whole body of the wildtype mice was exposed. In case infection might happen, give the mice drink water containing antibiotics (in our case, neomycin was used). Sucrose (5% w/v) may be added to drinking water for energy supplement, but the water should change daily in case bacterial may grow.
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bo Peng (bopeng@connect.hku.hk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The data that support the findings of this study are available from the corresponding author Bo Peng at Fudan University for reasonable request.

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AUTHOR CONTRIBUTIONS
B.P. and Y.R. conceived, designed, and conceptualized this study. B.P. supervised this study. Z.X., B.P., and Y.R. wrote the manuscript. Z.X. performed most of experiments. B.P. and Y.R. contributed to the interpretation of results. All authors discussed results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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