HMGB1 A Box binds to CXCR4 to inhibit HMGB1/CXCL12 mediating macrophage and T cell infiltration and prevents neuronal damage in Parkinson's Disease

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Abstract

Background and purpose: Our previous work proved that HMGB1 A Box significantly protected TH+ neurons in Parkinson's disease (PD) model mice and inhibited microglia activation and T cell infiltration including Th17 in the substantia nigra (SN). This study explored the mechanism of Th17 differentiation and how A Box inhibiting this process. Experimental approach: Clodronate liposomes were used to deplete the peripheral monocytes of mice, infused the labeled CD3+ T cells, and used immunoprecipitation to knock down the primary cells HMGB1 in vitro. At the same time, we combined the data of serum and blood cells of PD patients to study. Key results: Depletion of peripheral monocytes/macrophages reduced Th17 cell infiltration in the SN of MPTP mice and protected TH+ neurons. Co-culture experiments with knockdown of HMGB1 in primary cells showed that HMGB1, which induces monocyte/macrophage migration and infiltration into the SN, originates from neurons rather than glial cells. Data from MPP+-treated midbrain cell models and assays associated with adoptive transfer of CD3+ cells suggest that monocyte/macrophage and T cell migration into the SN is mediated by HMGB1/CXCL12-CXCR4. Co-immunoprecipitation and immunofluorescence confirmed that HMGB1 A Box bind to CXCR4 on T cells and macrophages, thereby competitively inhibiting their infiltration in SN. The HMGB1/CXCL12 complex is also present in the serum of PD patients. Conclusions and implications: HMGB1 A Box protects TH+ neurons by binding CXCR4 to inhibit the migration / infiltration of T cells and macrophages to SN mediated by HMGB1 / CXCL12 complex formed by neuron derived HMGB1.

1. Introduction

Since Parkinson's disease (PD) has been shown to be not only a degenerative but also an inflammatory disease of the central nervous system, evidence of $CD4^+$ T cell infiltration in the substantia nigra (SN) has been found in different PD animal models and PD patients(Baird *et al.* , 2019; Galiano-Landeira *et al.* , 2020; Subbarayan *et al.* , 2020). $CD4^+$ T cells and a subset of them Th17 cells was shown to have deleterious roles in PD(Bolte and Lukens, 2018; Dutta *et al.* , 2019; Liu *et al.* , 2019). HMGB1 plays an important role in the differentiation of $CD4^+$ T cells into Th17(Jhun *et al.* , 2015; Su*et al.* , 2011). Our previous study also confirmed that HMGB1 promotes the differentiation of $CD4^+$ T cells to Th17 in a microglia-dependent manner *in vitro* (Tian *et al.* , 2020). Peripheral monocyte/macrophage infiltration in SN of PD model animals is correlated with the course of PD(Harms *et al.* , 2018; Pillny *et al.* , 2021; Santaella *et al.* , 2020; Xie *et al.* , 2017). However, the specific role of these infiltrating peripherally derived monocytes/macrophages is unclear.

HMGB1 is an evolutionarily highly conserved nuclear protein that can be actively secreted by immune cells during stress or passively released after cellular injury(Andersson *et al.* , 2018; Zimmermann *et al.* , 2004). According to the different redox forms of the three cysteine sites of HMGB1, HMGB1 has a fully reduced form (frHMGB1), a disulfide bond form (dsHMGB1) and a fully oxidized form, with completely different

biological functions (Tian *et al.* , 2021). The deleterious role of elevated HMGB1 in PD has been repeatedly demonstrated(Santoro *et al.* , 2016; Sasaki *et al.* , 2016). frHMGB1 has chemotactic roles, however, whether HMGB1 plays a role in PD and whether frHMGB1 elicits the involvement of peripheral immune cells in neuroinflammation in PD remains to be explored.

Our previous data showed that HMGB1 A Box, a competitive inhibitor of HMGB1, had a very significant inhibitory effect on T cell infiltration including Th17 in the SN and in MPP^+-induced PD cells in vitro excluding the influence of peripheral immune cells. In the model, it was confirmed that the induction of Th17 by HMGB1 is dependent on microglia(Tian *et al.* , 2020). However, in this study, we found that microglia expressed significantly lower levels of MHC II than macrophages, it was reported that the presence monocyte/macrophage in the midbrain in an ∝-syn-induced mouse model(Choi *et al.* , 2020; Harms *et al.* , 2018; Tentillier *et al.* , 2016). Another study found an increase of HLA-DR expression on monocytes in cerebrospinal fluid and of $CD45RO^+$ T cells in peripheral blood in PD patients(Fiszer *et al.* , 1994). The peripheral monocytes may also promote Th17 differentiation in PD.

2. Materials and methods

2.1. Human samples

40 human serum samples (20 PD patients and 20 healthy people in the same age range) were obtained from Jiangsu Taizhou People's Hospital. The collection and use of all clinical samples were approved by the Clinical Research Ethics Committee of Taizhou People's Hospital. All patients and healthy people had no history of other inflammatory diseases and anti-inflammatory drugs within the past 2 months. Serum HMGB1 and CXCL12 were detected according to the protocol of the ELISA kits (Solarbio; MultiSciences). For the correlation analysis of clinical data, we used Pearson's chi squared ($\chi 2$) Test. $p < 0.05$ was considered statistically significant. For human serum co-immunoprecipitation, serum sample was first placed in 100kDa Amicon Ultra-15 (Millipore) and centrifuged at 1000rcf for 20 minutes to obtain IgG-depleted PD patient serum. Subsequently, the samples were incubated with anti-human HMGB1 monoclonal antibody (ab228624, Abcam) and Protein A+G agarose beads (P2012, Beyotime); after thorough washing, protein loading buffer was added to the agarose beads for a boiling water bath for 5 min, and then SDS-PAGE electrophoresis was performed, and CXCL12 was detected by antibody.

For the isolation of $CD3^+$ CD14+ cells from human peripheral blood, first centrifuge the peripheral blood at 500 rcf for 10 min, lyse the red blood cells with ACK, then resuspend the cells in buffer, incubate overnight in CXCR4 antibody or IgG from the same species, and add Protein A+G agar after centrifugation and washing. The sugar beads were incubated for 3 hours, and then centrifuged and washed several times. The agarose beads were reselected with protein loading buffer, and the beads were bathed in boiling water for 5 minutes, and then HMGB1 and CXCL12 was detected by SDS-PAGE electrophoresis.

2.2. Animal model

All experimental procedures were carried out in accordance with the animal experiment guidelines of Jiangsu University and were supervised and approved by the Animal Experiment Committee of Jiangsu University. 8˜10 weeks old male C57BL/6J mice weighing 22-25g were purchased from the Experimental Animal Center of Jiangsu University. All animals are housed in a room controlled by temperature and humidity and can eat and drink freely in a 12-hour light/dark cycle. For MPTP induction, mice were i.p. injected with MPTP dissolved in normal saline at a dose of 20 mg/kg per day for 7 days. For HMGB1 A Box, mice received recombinant A Box protein i.v. dissolved in saline for 7 days at a dose of 5 mg/kg for 7 days. The recombinant HMGB1 A Box is expressed and purified by our lab, and the steps refer to our previous work (Tian*et al.* , 2020). For clodronate liposomes (LIPSOMA), mice were i.v. one day in advance, a total of 3 times, 100μl per mouse each time, in order to fully deplete circulating monocytes. The other mice were injected with saline as control. 7 days later, after isoflurane anesthesia, all mice were perfused with PBS and the brains of mice were then obtained and further processed. Mice requiring brain slices were perfused with 4% paraformaldehyde after PBS perfusion. After obtaining the brain, the brain was fixed in paraformaldehyde, then dehydrated in 20% and 30% sucrose-PBS solution respectively, and then made

continuous frozen sections (30μm thick) of SN.

2.3. Immunohistochemical staining

7 days after MPTP injection, the animals were anesthetized with isoflurane, perfused with heparinized PBS solution through the heart, and then perfused with 4% paraformaldehyde and PBS solution. Then the whole brain was fixed overnight in 4% paraformaldehyde, and then in 20% and 30% sucrose solution prepared by PBS for 48 hours respectively. After that, the brain was frozen on ice and continuous coronal frozen sections were made with a thickness of 30 μm. The obtained brain tissue sections were placed in 50% glycerol/PBS solution and stored at -20 °C. For fluorescence staining, sections from the same position of SN of mice in each group were washed in PBS, blocked in 5% BSA, and then used with anti-F4/80 (cat#157304, Biolegend), anti-CD45 (cat#157214, Biolegend), anti-CD11c (cat#157305, Biolegend) or anti-CD31 (ab281583, Abcam) antibodies. Shake gently overnight at 4 °C. The next day, it was washed three times in PBS for 5min, and then applied to the appropriate secondary fluorescent antibody for 1 hour at room temperature. Then, the slices were installed on the coated glass slide and sealed with anti-fluorescence quencher.

For immunofluorescence of mouse and human T cells, monocytes and macrophages, after the purity of the cells sorted by magnetic beads was identified, they were fixed in a centrifuge tube by 4% paraformaldehyde for 10 minutes, and washed with PBS after centrifugation. CXCR4, 6×His, HMGB1, CXCL12 antibodies were added and stained overnight at 4°C. After 3-time washes with PBS, the fluorescent secondary antibodies were added for 1 h at room temperature in the dark, and washed 3 times with PBS. Resuspend in an appropriate volume of anti-fluorescence quencher. Finally, photographs were taken using a super-resolution fluorescence microscope.

2.4. Isolation of neurons and glial cells

For primary mesencephalic neurons, refer to the previous method (Han*et al.* , 2003). After the newborn C57BL/6 mice were anesthetized with isoflurane, the brain was taken out, and placed in cold DMEM culture medium, the midbrain was carefully cut off and the meninges and blood vessels were separated under stereological microscope, then digested into single cell suspension with 0.25% trypsin/PBS solution, precipitated and removed large impurities, and continued to be cultured in Neurobasal medium containing 2% B27. For primary microglia, after centrifuging the single cell suspension obtained by the above steps, use 10% FBS DMEM culture medium in the poly-L-lysine-coated culture plate, change the culture medium every 2 day, and continuously culture for 7-10d until the cells grow on the culture plate, until the microglia can be clearly seen attached to the surface of the underlying astrocytes under the microscope. Digest with 0.25% trypsin, observe that most microglia fall off under the microscope, terminate the digestion with culture medium, centrifuge the suspension containing microglia and culture it in DMEM medium containing 10% FBS for use. At the same time, identify the purity of microglia through Iba1 immunofluorescence to ensure that the proportion of $Iba1^+$ cells exceed 95%. For primary astrocytes, GFAP immunofluorescence showed that the proportion of $GFAP^+$astrocytes reached 90%.

2.5. Isolation of $CD3^+$ T cells and naïve $CD4^+$ T cells

After 8-week-old C57BL/6J mice were anesthetized with isoflurane, the spleen was taken out, and the spleen single-cell suspension was prepared following the previous method (Flaherty and Reynolds, 2015), and the spleen cells were treated following the standard protocol of Naïve $CD4^+$ T Cell Isolation Kit (19765A, STEMCELL) to isolate naïve $CD4^+$ T cells. Then they were cultured in a plate coated with CD3 antibody (100339, Biolegend), and continued to be cultured in the presence of CD28 antibody (102115, Biolegend) until be used. For GFP mouse spleen $CD3^+$ T cells, after obtaining spleen single cell suspension in the same manner as above, Biotin anti-mouse CD3 Antibody (100303, Biolegend) and Anti-Biotin MicroBeads (5190902231, Miltenyi Biotec) were added successively according to the product protocals. The LS column magnetic bead sorting method was used to obtain $CD3^+$ T cells with a purity of more than 90%.

2.6. Adoptive transfer of $CD3^+$ T cells

The spleen-derived GFP^+ $CD3^+$ T cells obtained by magnetic bead sorting were treated with or without 1

ug/mL CXCR4 neutralizing antibody and HMGB1 A Box for 24 h in vitro. Cells were then collected and transfused back into an 8-10 week old wild-type C57BL6J male mice with a source of cells. The recipients were then given daily intraperitoneal injection of MPTP 20 mg/kg for 7 days. After the mice were anesthetized with isoflurane on the 8th day, the mice were perfused with PBS and paraformaldehyde successively, and the mouse brains were taken and serially sectioned at the SN. CD3 and GFP fluorescence were observed under a microscope.

2.7. Flow cytometry

Cells from mouse midbrain or co culture system were stained by CD45, CD11c, CD11b, CD4, MHC II, IFN-γ, IL-4 and IL-17A flow cytometry antibody (Biolegend). For IFN- γ, IL-4 and IL-17A staining, the cells were treated with 25 ng/ml PMA (Sigma Aldrich) and 1 mg/mL ionomycin (Sigma) for 5h before cell collection. After surface antibody staining, the cells were fixed and permeabilized with FIX &PERM MEDIUM A and FIX &PERM MEDIUM B (multi-Sciences), and then stained with intracellular factors antibody at 4 . After the staining step, the cells were washed with PBS and analyzed by FCM.

2.8. Transwell migration assay

Transwell migration analysis was performed in 24-well Boyden chamber (diameter 6.5mm, pore diameter 8.0μm, CORNING). Mouse peritoneal macrophages were isolated by time difference adhesion method, and they were treated with the number of 1×10^4/well was seeded in the upper chamber. The macrophages in the upper chamber were treated by recombinant HMGB1 A Box protein to the final concentration of 200ng/ml or AMD3100 to the final concentration of 200ng/ml or CXCR4 neutralizing antibody to the final concentration of 5ug/ml; Primary cells from the midbrain with different treatments or combinations were placed in the lower chamber. After 24 hours of co-culture, the cells on the inner side of the upper chamber were gently removed. The cells on the lower side of the chamber were fixed with 4% paraformaldehyde, then stained with crystal violet, photographed under the microscope with 200 times magnification, and counted in 3 random fields with 400 times magnification.

2.9. Immunoprecipitation

For IP experiments detecting HMGB1 A Box/CXCR4 binding, the primary macrophages or $CD3^+$ T cells isolated by the differential adherence method were extracted with IP protein lysis solution, and a part of the protein solution was reserved as Input, and the rest protein was added to 40μL Protein A+G agarose beads (Beyotime, p2012) and incubated overnight at 4°C. Then, 1μg of CXCR4 monoclonal antibody (Abcam, ab181020) and 1μg of IgG from the same source as CXCR4 antibody were added and incubated for 3 hours at 4°C. Then 1 mL of IP lysis buffer was used to resuspend the agarose beads, centrifuge at 1000 rcf for 5 min, discard the supernatant, and repeat this step 5 times to fully wash unbound proteins. After last washing step, add 40μL of 1×protein loading buffer to the agarose beads, boiling water bath for 5-10min, SDS-PAGE electrophoresis. The corresponding protein bands were detected using CXCR4 and 6×His (Abcam, ab18184) antibodies.

For IP experiments to detect the binding of HMGB1/CXCL12, a part of the supernatant from MPP^+-treated midbrain cells was freeze-dried at low temperature, or a part of serum from PD patients whose serum immunoglobulins were removed by ultrafiltration through a 100kDa ultrafiltration tube as Input. And the rest protein was added to 40μL Protein A+G agarose beads and incubated overnight at 4°C. Then add 1μg of HMGB1 monoclonal antibody and 1μg of IgG from the same source of HMGB1 antibody (Abcam, ab190377) and incubate at 4°C for 3 hours. Then use 1 mL of IP lysis buffer to resuspend the agarose beads, centrifuge at 1000 rcf for 5 min, discard the supernatant, and repeat this step 5 times. After washing, add 40μL of 1×Loading Buffer to the agarose beads, centrifuge the agarose beads to the bottom, take a boiling water bath for 5-10 minutes, and perform SDS-PAGE electrophoresis. The corresponding protein bands were detected using HMGB1 and CXCL12 (ab25117) antibodies.

For the experiment to detect the binding of CXCR4 to HMGB1/CXCL12, the $CD14^+$ monocytes of PD patients obtained by magnetic bead sorting were added to IP lysate to extract the protein, and a part

was taken as Input. 1μg of CXCR4 monoclonal antibody and 1μg of IgG from the same source as CXCR4 antibody were addedto the remaining protein solution and incubated at 4°C for 3 hours. Then use 1mL of IP lysis buffer to resuspend the agarose beads, centrifuge at 1000 rcf for 5 min, discard the supernatant, and repeat this step 5 times to fully wash unbound proteins. After washing, add 1×Loading Buffer to the agarose beads, centrifuge the agarose beads to the bottom, take a boiling water bath for 5-10 minutes, and perform SDS-PAGE electrophoresis. The corresponding protein bands were detected using CXCR4, HMGB1 and CXCL12 antibodies.

2.10.Western blot

After PBS cardiac perfusion in isoflurane anesthetized mice, midbrain was separated and removed meninges and blood vessels under stereological microscope, and then tissue proteins were extracted by RIPA lysis buffer (P0013B, Beyotime). The protein concentration of the obtained protein was measured by BCA Protein Assay Kit (P0012S, Beyotime). Finally, the protein concentration between samples was adjusted to be uniform according to the BCA results, and the protein Loading buffer was added to the water bath 100 for 10min. Then the protein samples were separated in 10% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane were incubated in TBST solution containing TH (#45648, Cell Signaling Technology) and β-Actin (#3700, Cell Signaling Technology) primary antibodies overnight at 4 °C. After washing by TBST for 10min 3 times, PVDF membrane was incubated in HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse 1:10000, Abcam) for 2h at room temperature, after washing by TBST for 10min 3 times, BeyoECL Moon (P0018FS, Beyotime) solution was used for PVDF membrane chemiluminescence detection. Protein banding results were grayscale scanned by Image J.

3. Results

3.1. HMGB1 A Box inhibits macrophage infiltration in SN.

In previous studies, we detected that T cell infiltration in SN could be significantly inhibited by HMGB1 A Box, and *in vitro* experiments confirmed that midbrain microglia promoted Th17 differentiation. However, the proportion of microglia promoting Th17 differentiation*in vitro* was significantly lower than that of MPTP model mice. Therefore, we assume that peripheral antigen-presenting cells may also contribute to Th17 differentiation in SN. In this study, we detected $CD45^{+}F4/80^{+}$ macrophages and $CD45^{+}CD11c^{+}$ DCs in the SN of MPTP mice, and found that macrophages were outside the blood vessels or lymphatic vessels marked by CD31-positive endothelial cells, this indicates that macrophages are infiltrating the parenchyma of the SN, and HMGB1 A Box significantly inhibited this process (Fig. 1A). However, although MPTP also induced a certain increase in $CD45^{+}CD11c^{+}$ DCs, they were localized inside the blood vessels rather than the parenchyma (Fig. 1B).

3.2. HMGB1 A Box reduces Th17 and protects TH^{+}neurons by inhibiting monocytes/macrophages infiltration.

Clodronate liposome depleted peripheral monocyte/macrophage mice showed more TH^{+} neurons in the SN compared to MPTP induced mice, and at the protein level, this protective effect was similar to that of HMGB1 A Box (Fig. 2A, B). Subsequently, we performed flow cytometry on the above-mentioned mouse SN, and the gating strategy is shown in the figure (Fig. 2C). Injections of HMGB1 A Box and clodronate liposomes did not affect the proportion of $CD45^{int}CD11b^{+}$ microglia in the SN (Fig. 2D), but compared with the MPTP group, HMGB1 A Box and clodronate liposome injected mice showed fewer $CD45^{hi}CD11b^{+}$ cells in the SN (Fig. 2E). In contrast, clodronate liposome injected mice did not affect the proportion of $CD45^{hi}CD11c^{+}$ DCs in the SN compared with MPTP mice, although they were all elevated relative to the Control group (Fig. 2F). Among the three APCs detected in SN, microglia accounted for the highest proportion, followed by macrophages, while DCs had the significantly lowest (Fig. 2D, E and F). This suggests that the cells that mainly present antigens to T cells in PD model mice are less likely to be DCs.

We also detected the MHC II levels of these three APCs to assess their potential antigen-presenting capacity. There was no significant difference in the expression levels of MHC II among the above three cells in the SN

of mice between each treatment group, suggesting that our previous finding that HMGB1 A Box inhibits Th17 levels in the SN of PD model mice may be irrelevant with affecting the antigen presentation ability of APCs in the SN (Fig. 2G, H and I). Comparing the differences between the three APCs, it can be seen that DCs have the highest expression levels of MHC II, microglia the lowest, and monocytes/macrophages have intermediate levels of MHC II expression (Fig. 2G, H and I). In the SN, Th2 and Th17 were elevated in the MPTP group, and both population-level inhibition was shown in both HMGB1 A Box and clodronate liposome injected mice, and Th17 was the main subset among the three Th cell subsets Th1, Th2, and Th17 (Fig. 2J, K and L).

3.3. Peripheral monocytes/macrophages promote Th17 differentiation in MPTP-induced mice.

Although depletion of monocyte-macrophages with clodronate liposomes reduced the number of Th17, and application of HMGB1 A Box also reduced the monocytes/macrophages in the SN and ultimately reduced the number of Th17, it cannot be confirmed *in vivo* whether microglia and monocytes/macrophages promote the proliferation, migration of Th17 or the differentiation and of naïve $CD4^+$ T cells into Th17. Therefore, we took the midbrain of mice in the MPTP model group or MPTP+A Box group, stripped the meninges and blood vessels, and cultured them *in vitro* . Since adult mouse neurons are difficult to survive, each group was supplemented with midbrain neurons isolated from neonatal mice, and pre-stimulated mouse spleen naïve $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies were added to this co-culture system (Fig.3A). Groups are: Control group (normal mouse midbrain cells + neonatal mouse midbrain neurons + naïve $CD4^+$ T cells), MPTP group (MPTP model mouse midbrain cells + neonatal mouse midbrain neurons + naïve $CD4^+$ T cells), MPTP-microglial group (MPTP model mice with microglia-depleted midbrain cells + neonatal mouse midbrain neurons + naïve $CD4^+$ T cells), MPTP+A Box group (HMGB1 A Box intervened with MPTP mouse midbrain cells + neonatal mouse midbrain Neurons + naïve $CD4^+$ T cells) and MPTP+A Box-microglia group (microglia-depleted midbrain cells from HMGB1 A Box treated MPTP mice + neonatal mouse midbrain neurons + naïve $CD4^+$ T cells) (Fig.3B), since the addition of naïve $CD4^+$ T cells, the entire co-culture system was maintained for 72h, and then all cells were collected and detected by FCM for CD45, CD11b, CD4, and IL-17A, as shown in the circle gate (Fig.3C). The results showed that the proportion of $CD45^{hi}$ $CD11b^+$monocytes/macrophages in MPTP group was the highest, compared with that in MPTP+A Box group, $CD45^{hi}CD11b^+$monocytes/macrophages decreased significantly (Fig. 3F); Consistent with this, the proportion of Th17/$CD4^+$ T cells in MPTP group was the highest, while that in MPTP + A Box group was significantly lower (Fig. 3D, G), indicating that monocytes/macrophages play an important role in promoting Th17 differentiation. In MPTP microglial group and MPTP+A Box microglial group, after removing microglia (Fig. 3E), the proportion of Th17/$CD4^+$ T cells also decreased significantly (Fig. 3D,G), indicating that microglia can also promote Th17 differentiation in this system.

3.4. HMGB1 A Box binds CXCR4 to inhibit HMGB1/CXCL12 complex-mediated infiltration of monocytes/macrophages and T cells into the SN.

Mario Tirone *et al* . previously reported that the fully reduced form of HMGB1 (frHMGB1) binds CXCR4 of myofibroblasts to induce their migration and promote tissue repair(Tirone *et al.* , 2018).*In vitro,* cell experiments by Milena Schiraldi *et al* . confirmed that the complex formed by HMGB1 and CXCL12 significantly induced the migration of human monocytes(Schiraldi *et al.* , 2012). Given that monocytes/macrophages infiltration in SN was significantly inhibited by HMGB1 A Box in our study, it is possible that HMGB1 A Box inhibited monocytes/macrophages migration by targeting this pathway. Therefore, intact midbrain cells from newborn C57BL/6J mice were isolated and co-cultured with peritoneal macrophages in a transwell system, and the results showed that MPP^+-treated mixed cultures of midbrain cells caused the greatest degree of macrophage migration, whereas HMGB1 A Box, HMGB1 neutralizing antibody, CXCR4 neutralizing antibody, or AMD3100-treated cultures all showed significantly less migration of macrophages. This suggests an important role for HMGB1, CXCL12 and CXCR4 in the MPTP/MPP^+-based PD model (Fig.4 A, B).

Compared with CXCL12 alone, the chemotaxis of HMGB1 complexed with CXCL12 on CXCR4-transfected cells was enhanced more than a hundred-fold(Schiraldi *et al.* , 2012). HMGB1/CXCL12-CXCR4 may also exist in the MPP^+-induced PD cell model. The co-immunoprecipitation experiments of HMGB1 and

CXCL12 in the supernatants of MPP^+-treated midbrain cells showed that HMGB1/CXCL12 complexes existed in this *in vitro* co-culture system (Fig. 4D). And after primary macrophages were cultured in the supernatant of MPP^+-treated midbrain cells, CXCR4 on macrophages was detected to bind HMGB1 and CXCL12 (Fig. 4E). As a domain of HMGB1, HMGB1 A Box has receptor binding potential similar to HMGB1, so we used co-immunoprecipitation and immunofluorescence to evaluate the possibility of HMGB1 A Box competitively binding to CXCR4 on macrophages. The result showed that HMGB1 A Box partially co-localized with CXCR4 (Fig. 4C), and HMGB1 A Box-6xHis was pulled down using a CXCR4 monoclonal antibody. However, to exclude influence by the 6xHis tag, HMGB1 B Box-6xHis, as a control, was not detected, which indicated that HMGB1 A Box indeed binds to CXCR4 on macrophage (Fig. 4F).

Neurons, microglia, and astrocytes in the SN during PD are the source of elevated HMGB1 (Sasaki *et al.* , 2016). To determine the main source of HMGB1 which causes cell migration in PD, midbrain neurons, microglia and astrocytes from neonatal mice were isolated and cultured, and after knockdown of intracellular HMGB1 using lentivirus, different combined ways of these cells were co-cultured with peritoneal macrophages in the lower chambers of transwell system, and MPP^+ treatment was added to the lower chamber to induce neuronal damage at the same time. It was found that the mixed culture of all the three cells without knockdown of HMGB1 had the strongest effect on the migration of macrophages. and the application of AMD3100, an antagonist that specifically inhibits CXCR4-CXCL12 binding, significantly inhibited the migration of macrophages, and in the mic+ast+neu^{si} group which knockdown neuronal HMGB1, the number of macrophage migration was also significantly reduced (Fig. 4G, H). This indicates that HMGB1 released by midbrain neurons during PD plays an essential role in the migration of peripheral monocytes to the SN.

3.5. HMGB1 A Box inhibited T cell infiltration partially through binding of CXCR4 on T cells.

T cells express CXCR4 like macrophages. Given that CXCR4 mediates HMGB1-induced macrophage migration and infiltration into SN, we wanted to explore whether CXCR4 also mediates T cell migration and infiltration during PD and whether HMGB1 A Box also combines with CXCR4 to play a competitive inhibitory role. Firstly, mouse spleen $CD3^+$ T cells were isolated and cultured, and HMGB1 A Box was added to the culture for 6h. Cell immunofluorescence staining and co-immunoprecipitation data showed that HMGB1 A Box could bind to CXCR4 on T cells (Fig. 6A, B). To evaluate whether targeting CXCR4 on the surface of T cells inhibits T cell migration, spleen $CD3^+$ T cells from GFP mice were isolated, treated with HMGB1 A Box or CXCR4 neutralizing antibody *in vitro* , washed extensively, and then transfused into wild-type mice. The PD model was then induced with MPTP. Finally, immunofluorescence results in mouse SN showed that compared with mice reinfused with control $CD3^+$ T cells, HMGB1 A Box and CXCR4 neutralizing antibody-treated $CD3^+$ T cells transfused mice had significantly less GFP^+CD3^+ T cells infiltrated in the SN (Fig. 6D), and more GFP^+CD3^+ T cells remained in the periphery (Fig. 6C). These data suggest that CXCR4 of T cells also mediates T cell infiltration into the SN in MPTP induced PD model, and this process can be directly inhibited by the binding of HMGB1 A Box to CXCR4.

3.6. HMGB1/CXCL12 complex in the serum of PD patients.

Since the HMGB1/CXCL12 complex plays an important role in the infiltration of monocytes/macrophages and T cells in a mouse model. Previously, it has also been reported that the up-regulation of CXCL12 and HMGB1 in PD patients may be related to the course of PD(Li *et al.* , 2019; Santoro *et al.* , 2016). However, there is no evidence whether the HMGB1/CXCL12 complex exists in PD patients. We detected the serum levels of HMGB1 and CXCL12 in 20 PD patients and 20 healthy people in the same age range. The results showed that PD patients had significantly higher serum HMGB1 and CXCL12 levels (Fig. 6A, B). Moreover, PD patients showed a significant positive correlation between HMGB1 and CXCL12 levels, which highly suggested a possible combination between the two (Fig. 6C). Serum co-immunoprecipitation experiments also confirmed the existence of the HMGB1/CXCL12 complex (Fig. 6D). There is a certain relationship between PD and age. The degeneration of dopaminergic neurons when aging is one of the causes of PD. When we analyzed the correlation between HMGB1 and CXCL12 in PD patients and age, a significant negative correlation was observed between CXCL12 and PD patients' age, while HMGB1 levels

were not significantly correlated with age in PD patients (Fig. 6E, F). Then, we used magnetic beads to sort $CD3^+$ T cells and $CD14^+$monocytes from the peripheral blood of PD patients, and the results of co-immunoprecipitation showed that CXCR4 of CD14 monocytes bound to both HMGB1 and CXCL12 (Fig. 6G). And immunofluorescence results showed that HMGB1 and CXCL12 co-localized with CXCR4 on the surface of $CD3^+$ T cells and $CD14^+$ monocytes (Fig. 6H). These results of patients may suggest that treatment targeting HMGB1/CXCL12-CXCR4 may be more suitable for patients with earlier Parkinson's disease.

4. Discussion

HMGB1 is significantly up-regulated in PD model animals and patients, and its' up-regulation is closely related to PD. The use of HMGB1 monoclonal antibody and a variety of compounds that inhibit HMGB1 have observed protective effects on TH^+ neurons in PD model animals(Huh *et al.* , 2011; Santoro *et al.* , 2016; Sasaki*et al.* , 2016). Deficiency of HMGB1 downstream receptor TLR4 also protected TH^+ neurons in a mouse model of MPTP (Campolo *et al.* , 2019). TLR4 are mainly expressed on the surface of microglia in the CNS and mediate the pro-inflammatory effects of glial cells (Rahimifard *et al.* , 2017). Among the three redox forms of HMGB1, only the dsHMGB1 has the ability to bind to TLRs (Venereau *et al.* , 2012; Yang *et al.* , 2010; Yang *et al.* , 2015). The evidence seems to suggest that in PD, dsHMGB1 is the HMGB1 that plays a deleterious role. However, due to frHMGB1 will always oxidize into dsHMGB1 in inflammatory state(Palmblad *et al.* , 2015), laboratory evidence for the role of frHMGB1 in PD is still few. Previously, it was shown that injection of frHMGB1 into the brain of healthy rats was sufficient to induce blood-brain barrier disruption and IL1-β production(Aucott *et al.* , 2018). In PD, the passive release of frHMGB1 in the nucleus of persistently injured neurons likely plays an important role in the negative effects of HMGB1. Given that knockdown of neuronal HMGB1 affects neuronal development *in vivo* , our*in vitro* knockdown of midbrain neuron HMGB1 confirmed this.

Recently, Th17 cell subsets have revealed their deleterious effects in PD in both animal models and patient samples(Chandra *et al.* , 2016; Dutta *et al.* , 2019; Elgueta *et al.* , 2019; Reynolds*et al.* , 2010). We previously found that HMGB1 A Box has a surprising inhibitory effect on T cell infiltration and Th17 differentiation in addition to protecting TH^+ neurons and inhibiting glial activation(Tian *et al.* , 2020). Although the stereotactic injection of exogenous frHMGB1 into the rat brain can't increase the expression of phagocyte MHC II like the rats injected with dsHMGB1, it is also enough to lead to the destruction of blood-brain barrier and the increase of $CD68^+$ phagocytes(Aucott*et al.* , 2018). frHMGB1, passively released from the nucleus during dopaminergic neuron damage, may play a key role in the recruitment of peripheral immune cells.

Our results showed that $CD45^+$ $F4/80^+$peripherally derived macrophages were infiltrated in the SN of the MPTP mouse model and were significantly reduced after HMGB1 A Box application. Previously, significant nuclear-cytoplasmic HMGB1 translocations from neurons, astrocytes and microglia were observed in the PD mouse MPTP model(Santoro *et al.* , 2016). However, the roles of these different cell-derived HMGB1 on immune cells in the SN of PD model animals lacked precise definition. We found that in the MPP^+ treated primary cell co-culture system, knockdown of HMGB1 in midbrain neurons had the most significant inhibitory effect on macrophage. This suggests that HMGB1 released by sustained injury of neurons plays a crucial role in the recruitment of peripheral monocyte/macrophages. The direct inhibitory effect of HMGB1 A Box on macrophage and T cell was confirmed by experiments with HMGB1 A Box-treated macrophages transwell experiment and transfusion of HMGB1 A Box-treated GFP^+CD3^+ T cells into wild type mice. Co-immunoprecipitation and immunofluorescence confirmed that HMGB1 A Box was able to bind to CXCR4 on the surface of these cells. Mice reinfused with CXCR4-neutralizing antibody-treated GFP^+CD3^+ T cells had significantly fewer GFP^+ cells in the SN after 7 day-MPTP induction, although treatment with neutralizing antibody *in vitro* did not inhibit the CXCR4 expression of proliferated cells of GFP^+CD3^+ T cells *in vivo* . But it was also sufficient to confirm that the migration and infiltration of T cells into the SN was at least partially dependent on CXCR4. Ans in our previous study, we found that after the application of HMGB1 A Box, the levels of HMGB1 and CXCL12 were both reduced, and the infiltration of $CD3^+$ T cells in the SN

was almost disappear(Tian*et al.* , 2020). This also suggests that the HMGB1/CXCL12-CXCR4 axis plays an important role in T cell infiltration in PD.

Our previous study revealed the important role of microglia in the differentiation of Th17. In a pure *in vitro* co-culture system that excludes the influence of peripheral immune cells, naïve $CD4^+$ T cells will not be able to differentiate into Th17 without the presence of microglia. This time, we used an *in vivo* model to confirm that peripheral monocytes also have a significant effect on Th17 differentiation in the SN in MPTP mouse model. Several*in vitro* researches reported that the combination of CXCR4/CXCL12 induces the migration of macrophages, DCs and even microglia (Campana*et al.* , 2009; Schiraldi *et al.* , 2012; Tanabe *et al.* , 1997). In contrast, CXCL12 needs to form a complex with HMGB1 to exert its strong migration-inducing effect(Schiraldi *et al.* , 2012). In this study, for the first time, we detected the presence of HMGB1/CXCL12 in an *in vitro* model of MPP^+induced mouse midbrain cells, and also found evidence that HMGB1 A Box binds to the complex receptor CXCR4.

Previous studies reported an overall increase in CXCR4 and CXCL12 levels in the SN during PD(Shimoji *et al.* , 2009). In this study, we detected increased HMGB1 and CXCL12 in the serum of PD patients. Excitingly, we also found that the HMGB1/CXCL12 complex also exists in the serum of PD patients, and observed binding of CXCL12 and HMGB1 to CXCR4 on patients' peripheral blood $CD14^+$ monocytes and $CD3^+$ T cells. Before, it is reported that CXCR4 was up-regulated in peripheral blood mononuclear cells of PD patients(Bagheri *et al.* , 2018). This proved that the HMGB1/CXCL12-CXCR4 axis related mechanism is also involved in human PD. In addition, *in vitro* experiments have long confirmed that CXCL12 induces microglial migration(Tanabe *et al.* , 1997), and recently, studies have confirmed that CXCR4 on the surface of microglia in the SN of A53T mice is also up-regulated(Li *et al.* , 2019). This suggests that when dopaminergic neurons are damaged, surrounding microglia may also aggregate to the injury site through the HMGB1/CXCL12-CXCR4 axis, but this hypothesis still needs more experimental evidence to confirm. Interestingly, we found that CXCL12 was significantly negatively correlated with the age of PD patients, suggesting that targeting the HMGB1/CXCL12-CXCR4 axis in PD could be more suitable for less old patients.

In conclusions, our results suggest that neuron-derived HMGB1 plays an important role in the recruitment of peripheral monocyte-macrophages and T cells in PD model mice, and peripheral monocytes/macrophages infiltration in the SN also affects Th17 infiltration and differentiation. The application of HMGB1 A Box significantly inhibited the infiltration of peripheral monocytes/macrophages and T cells in the SN by binding CXCR4 (Fig. 7). This effect may make this protein of great clinical value at least in the treatment of some PD classification, which is mainly characterized by neuroinflammation.

Data Availability Statements

The data underlying this article are available in the article.

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Conflicts of Interest

The authors have declared that no conflict of interest exists.

Author contributions

Y. Tian, J. Jin, S. Zhang, Y. Cao, L. Xia, F. Liu performed experiment, analyzed, and interpreted data. S. Chakrabarti edited the manuscript. Z. Su wrote and designed the project.

Ethics approval

This study involves human participants and was approved by Clinical Research Ethics Committee of Taizhou People's Hospital (ID: Prot. Number KY 2022-002-01).

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Figure legends

Fig.1 HMGB1 A Box reduces macrophage infiltration in SN parenchyma. (A and B) Brains of animals receiving saline, MPTP, MPTP + HMGB1 A Box injection were separated and made into serial frozen sections of 30μm thickness were prepared at the end of the 7-day treatment, and sections of the SN site were subjected to CD45, F4/80, CD11c, CD31 immunofluorescence staining.

Fig.2 HMGB1 A Box reduces macrophages and Th17 infiltration in SN and protecting TH^+ neurons. (A and B) Brains of animals receiving saline, MPTP, MPTP + HMGB1 A Box, MPTP + clodronate liposomes were separated after 7 days of treatment and serial frozen sections were made and then

the sections of SN site were subjected to TH immunization group chemical staining, extract the protein from the SN for western blot detection of TH, and finally, the gray value of the results is counted (n=3; unpaired t-test; mean ± s.d.). (C) Digest mouse SN cells to obtain single cell suspension, and perform flow cytometry detection of CD45, CD11b, CD11c, MHC II, CD3, CD4, IFN-γ, IL-4 and IL-17A. (D-F) Proportion of $CD45^{int}CD11b^{+}$ microglia, $CD45^{hi}CD11b^{+}$ macrophages and $CD45^{hi}CD11c^{+}$ DCs in mouse SN (n=5; unpaired t-test; mean ± s.d.). (G-H) MHC II mean fluorescence intensity of $CD45^{int}CD11b^{+}$ microglia, $CD45^{hi}CD11b^{+}$ macrophages and $CD45^{hi}CD11c^{+}$ DCs in SN (n=5; unpaired t-test; mean ± s.d.). (J) Numbers of $CD4^{+}IFN\text{-}\gamma^{+}$ cells, $CD4^{+}IL\text{-}4^{+}$ cells and $CD4^{+}IL\text{-}17A^{+}$ cells in SN (n=3; unpaired t-test; mean ± s.d.). *P < 0.05, **P < 0.01.

Fig.3 Peripheral monocytes/macrophages promote MPTP-induced Th17 differentiation in mouse SN and are inhibited by HMGB1 A Box. (A) Mouse MPTP induction and midbrain cell isolation and culture timeline. (B) Mouse primary cells from the midbrain and $CD4^{+}$ naïve T cells co-culture groups. (C) Co-cultured cells were collected for CD45, CD11b, CD4 and IL-17A flow cytometry assays. (D and G) Ratio of $IL\text{-}17A^{+}$ cells to $CD4^{+}$ cells in each group of cells (n=4; unpaired t-test; mean ± s.d.). (E) Number of $CD45^{int}CD11b^{+}$ microglial in each group (n=4; unpaired t-test; mean ± s.d.). (F) Number of $CD45^{hi}CD11b^{+}$ macrophages in each group (n=4; unpaired t-test; mean ± s.d.). *P < 0.05, **P < 0.01.

Fig.4 HMGB1 complexes with CXCL12 mediate macrophage migration.(A and B) Peritoneal macrophages were co-cultured with MPP^{+}-treated primary midbrain cells in transwell system (n=4 random high-magnification fields of view; unpaired t-test; mean ± s.d.). (C) Immunofluorescence staining of 6×His and CXCR4 after 6 hour-HMGB1 A Box treatment of peritoneal macrophages. (D) After the culture supernatant of MPP^{+}-treated midbrain cells was concentrated, the binding of HMGB1/CXCL12 was detected by immunoprecipitation assay. (E) Mouse peritoneal macrophages were cultured with MPP^{+} -treated midbrain cell supernatant for 6 hours, and the binding of CXCR4 to HMGB1 and CXCL12 was detected by immunoprecipitation assay. (F) Peritoneal macrophages were treated with HMGB1 A Box for 6 hours, and the binding of HMGB1 A Box-6×His and CXCR4 were detected by immunoprecipitation assay. (G and H) Midbrain microglia (mic), astrocytes (ast) and neurons (neu) HMGB1 were knocked down separately and co-cultured with peritoneal macrophage in transwell system in different combinations, and cells in the lower chamber were treated with MPP^{+} (n=4 random high-magnification fields of view; unpaired t-test; mean ± s.d.). ***P < 0.005, ****P < 0.001

Fig.5 CXCR4 of $CD3^{+}$ T cell mediates their infiltration in the SN. (A) Immunofluorescence staining of 6×His and CXCR4 after HMGB1 A Box-6×His treatment of magnetic bead-sorted $CD3^{+}$ T cells for 6h. (B) $CD3^{+}$ T cells were treated with HMGB1 A Box for 6 hours, and the binding of HMGB1 A Box-6×His and CXCR4 were detected by immunoprecipitation assay. (C) FCM detection in peripheral blood of MPTP mice that were transfused with $CD3^{+}$ T cells from GFP mice (n=3; unpaired t-test; mean ± s.d.). (D) GFP and CD3 tissue immunofluorescence staining of MPTP mouse SN which were transfused with $CD3^{+}$ T cells from GFP mice. *P < 0.05, **P < 0.01,

Fig. 6 HMGB1/CXCL12-CXCR4 exists in the serum of PD patients.(A and B) Serum HMGB1 and CXCL12 levels of PD patients and healthy people were detected by ELISA. (C) Correlation analysis between serum HMGB1 levels and CXCL12 levels of PD patients. (D) Co-immunoprecipitation experiments of HMGB1 and CXCL12 in serum of PD patients. (E) Correlation analysis of serum HMGB1 level and age in PD patients. (F) Correlation analysis between serum CXCL12 level and age in PD patients. (G) Binding of HMGB1 and CXCL12 to CXCR4 in PD patient $CD14^{+}$ monocytes by co-immunoprecipitation. (H) Immunofluorescence detection of CXCR4, HMGB1 and CXCL12 on $CD3^{+}$ T cells and $CD14^{+}$ monocytes in PD patients. (n=20; unpaired t-test; mean ± s.d.) *P<0.05, **P<0.01.

Fig. 7 HMGB1 A Box suppresses immune cell responses in the SN of PD mice and its mechanism. After neuronal damage in SN, frHMGB1 passively released from neurons binds to CXCL12 in the surrounding environment to form frHMGB1/CXCL12 complex. This complex can bind to CXCR4 on the surface of nearby microglia, macrophages in blood or lymphatic capillaries and T cells to induce their

migration and infiltration into the SN parenchyma. The macrophages and microglia infiltrated in the SN jointly promote T cells to differentiate into Th17 and continue to damage neurons. Intravenous injection of HMGB1 A Box competitively inhibited this process by binding CXCR4 of these peripheral immune cells.