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Lactoferrin is required for early B cell development in C57BL/6 mice

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Abstract

Lactoferrin (Lf) is widely distributed in mammalian milk, various tissues, and their exocrine fluids and has many physiological functions, such as bacteriostasis, antivirus, and immunoregulation. Here, we provide evidence that lactoferrin is required for early stages of B cell development in mice. Lactoferrin-deficient ($Lf^{-/-}$) C57BL/6 mice showed systematic reduction in total B cells, which was attributed to the arrest of early B cell development from pre-pro-B to pro-B stage. Although the $Lf^{-/-}$ B cell "seeds" generated greater pro-B cells comparing to wild type (WT) littermates, the $Lf^{-/-}$ mice bone marrow had less stromal cells, and lower CXCL12 expression, produced a less favorable "microenvironment" for early B cell development. The underlying mechanism was mediated through ERK and AKT signalings and an abnormality in the transcription factors related to early differentiation of B cells. The $Lf^{-/-}$ mice also displayed abnormal antibody production in T cell-dependent and T cell-independent immunization experiments. In a pristane-induced lupus model, $Lf^{-/-}$ mice had more serious symptoms than WT mice, whereas lactoferrin treatment alleviated these symptoms. This study demonstrates a novel role of lactoferrin in early B cell development, suggesting a potential benefit for using lactoferrin in B cell-related diseases.

Keywords: Lactoferrin, B cell development, CXCL12, Pre-pro-B cell, Pro-B cell, Systemic lupus erythematosus

To the editor,

Lactoferrin (LF) is widely existed in mammalian milk, neutrophils particles and various tissues and their exudates. It plays a protective role of the body through a variety of functions, such as anti-infection, anti-oxidation, and immune modulation [1, 2]. By constructing a *lactoferrin* gene knockout ($Lf^{-/-}$) C57BL/6 mice [3, 4], here we investigated the effect of lactoferrin deficiency on B cell development.

Proportion of $CD45^+$, T, and dendritic cells remained fairly consistent, but proportion of total B cells of $Lf^{-/-}$ mice were significantly lower than that of WT controls (Fig. 1a, Additional file 2 Fig. S1). Lactoferrin deficiency did not cause an increase of B cell apoptosis (Fig. 1b). Proportion of hematopoietic progenitor cells displayed little difference between WT and $Lf^{-/-}$ (Fig. 1c, d). Proportion of pro-B, pre-B and immature B cells in bone marrow of $Lf^{-/-}$ mice were all significantly lower, whereas proportion of pre-pro-B cells was higher than that of WT (Fig. 1e, f), implying that lactoferrin deficiency inhibited the transition from pre-pro-B to pro-B stage. mRNA expression levels of *lactoferrin* are dynamic in developing B cells, peaking at the pre-pro-B stage (Fig. 1g).

Contrary to our expectations, the capability of pre-pro-B to generate pro-B cells was significantly higher in $Lf^{-/-}$ group than WT both in vitro (Fig. 1h, i) and in vivo (Fig. 1j, Additional file 3 Fig. S2), implying that $Lf^{-/-}$ "seeds" generated more pro-B cells comparing to WT "seeds". We analyzed the global transcriptome change of pre-pro-B and pro-B cells from WT and $Lf^{-/-}$ mice. GO, KEGG, and GSEA [5] reveal the signaling differences

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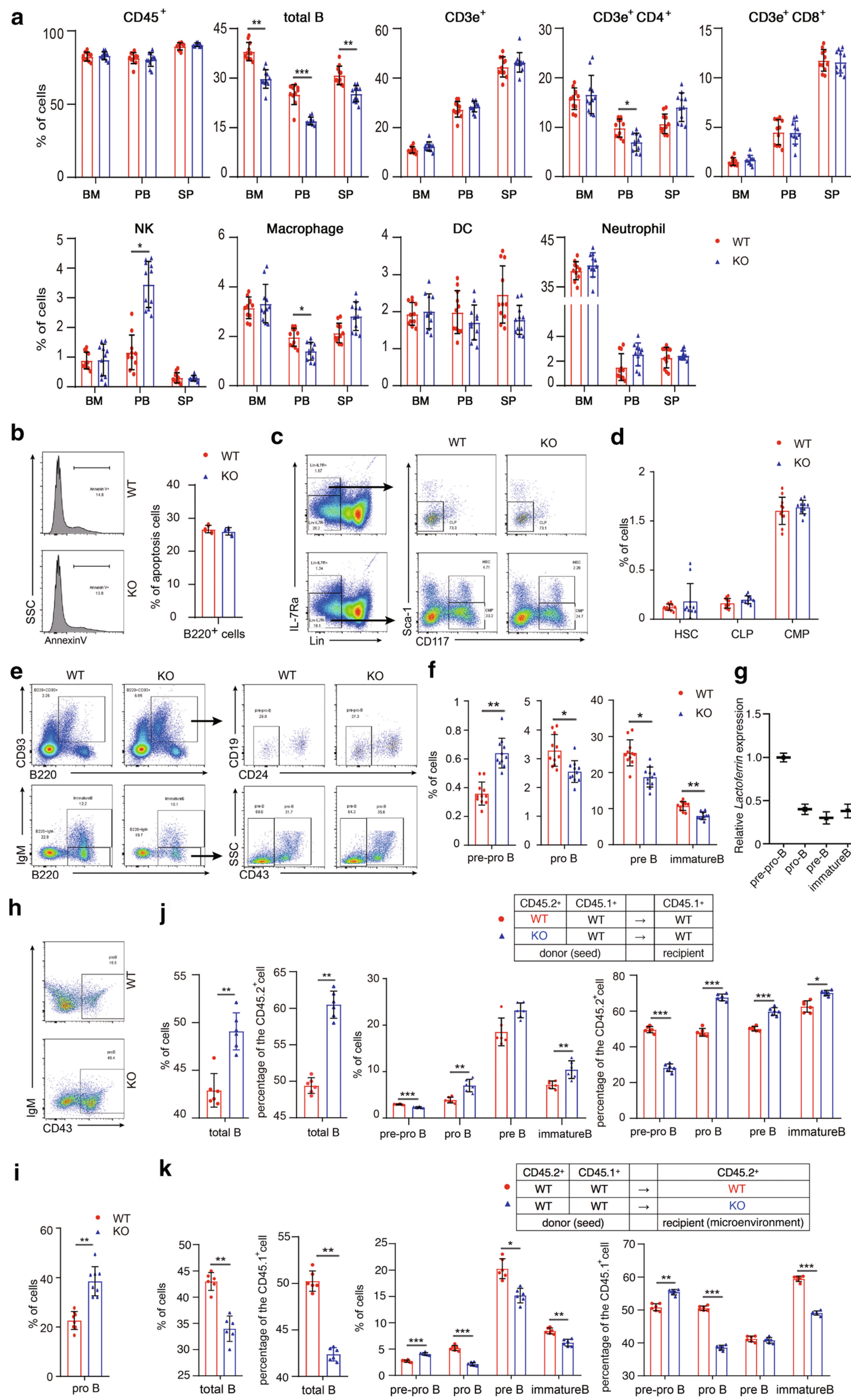

a
CD45+
total B
CD3e+
CD3e+ CD4+
CD3e+ CD8+
% of cells
BM PB SP
NK
Macrophage
DC
Neutrophil
WT
KO
b
SSC
AnnexinV
% of apoptosis cells
B220+ cells
c
IL-7Ra
Lin
Sca-1
CD117
d
HSC CLP CMP
e
CD93
B220
CD19
CD24
IgM
SSC
CD43
f
pre-pro B
pro B
pre B
immatureB
g
Relative Lactoferrin expression
h
i
j
CD45.2+ CD45.1+ CD45.1+
donor (seed) recipient
percentage of the CD45.2+cell
k
CD45.2+ CD45.1+ CD45.2+
recipient (microenvironment)
percentage of the CD45.1+cell

(See figure on previous page.)

Fig. 1 The defect of B cell development in *Lf*$^{-/-}$ mice is both cell autonomous and is associated with the bone marrow microenvironment. **a** Lactoferrin deficiency leads to imbalance of hematopoiesis. Cells were isolated from the bone marrow (BM), peripheral blood (PB), and spleens (SP) of *Lf*$^{-/-}$ mice and WT littermates. Frequencies of indicated cells were identified by flow cytometry. All immune cells were firstly gated on CD45$^+$. Each group has 11 mice. **b** Splenic B cells were sorted from WT and *Lf*$^{-/-}$ mice. The amount of apoptosis cells was detected by flow cytometer. Representative data from three independent experiments are shown. **c**, **e** Representative strategy of flow analysis of **c** HSC, CLP, CMP and **e** pre-pro-B, pro-B, pre-B, immature B cells. Cells were isolated from the mice bone marrow. **d**, **f** Frequencies of **d** HSC (Lin$^-$ IL7R$^-$ C-kit$^+$ Sca-1$^+$), CLP (Lin$^-$ IL7R$^+$ C-kit^{lo} Sca-1^{lo}), CMP (Lin$^-$ IL7R$^-$ C-kit$^+$ Sca-1$^-$) and **f** pre-pro-B (AA4.1$^+$B220$^+$CD19$^-$CD24$^-$), pro-B (B220$^+$CD43$^+$IgM$^-$), pre-B (B220$^+$CD43$^-$IgM$^-$), immature B (B220$^+$IgM$^+$) cells were identified by flow cytometry. Each group has 11 mice. (note, CD117 is C-kit, CD93 is AA4.1.) **g** mRNA expression of *lactoferrin* in distinct stages of developing B cells from WT mouse bone marrow was evaluated by RT-qPCR. **h**, **i** In vitro B cell differentiation experiment: purified pre-pro-B cells from *Lf*$^{-/-}$ or WT mice were cocultured with OP9 stromal cells in the presence of IL-7 (10 ng/ml), SCF (5 ng/ml), and Flt3L (5 ng/ml) for 9 days. **h** Representative data from eight specimens each group are shown. **i** The proportions of pro-B cells generated were then determined by flow cytometric analysis. **j** Bone marrow transplantation experiment: bone marrow cells from either WT or *Lf*$^{-/-}$ (CD45.2$^+$) mice with bone marrow from syngenic mice (CD45.1$^+$) were mixed at a 1:1 ratio. The recipient WT mice (CD45.1$^+$) were irradiated in fractionated doses (5 Gy × 2), and 16 h later, the recipient mice were injected with mixed cells (2 × 10^6 cells). After 6 weeks, the recipient mice were killed to prepare the bone marrow single-cell suspension, and the B cell proportion of each stage of B cell differentiation was analyzed by flow cytometry. Representative data from six mice each group are shown. **k** Bone marrow transplantation experiment: bone marrow cells from CD45.2$^+$ WT mice or from CD45.1$^+$ WT mice were mixed at a 1:1 ratio, while WT or *Lf*$^{-/-}$ mice with CD45.2$^+$ genetic background were used as recipient mice. The rest of the operation was the same as j. Representative data from six mice each group are shown

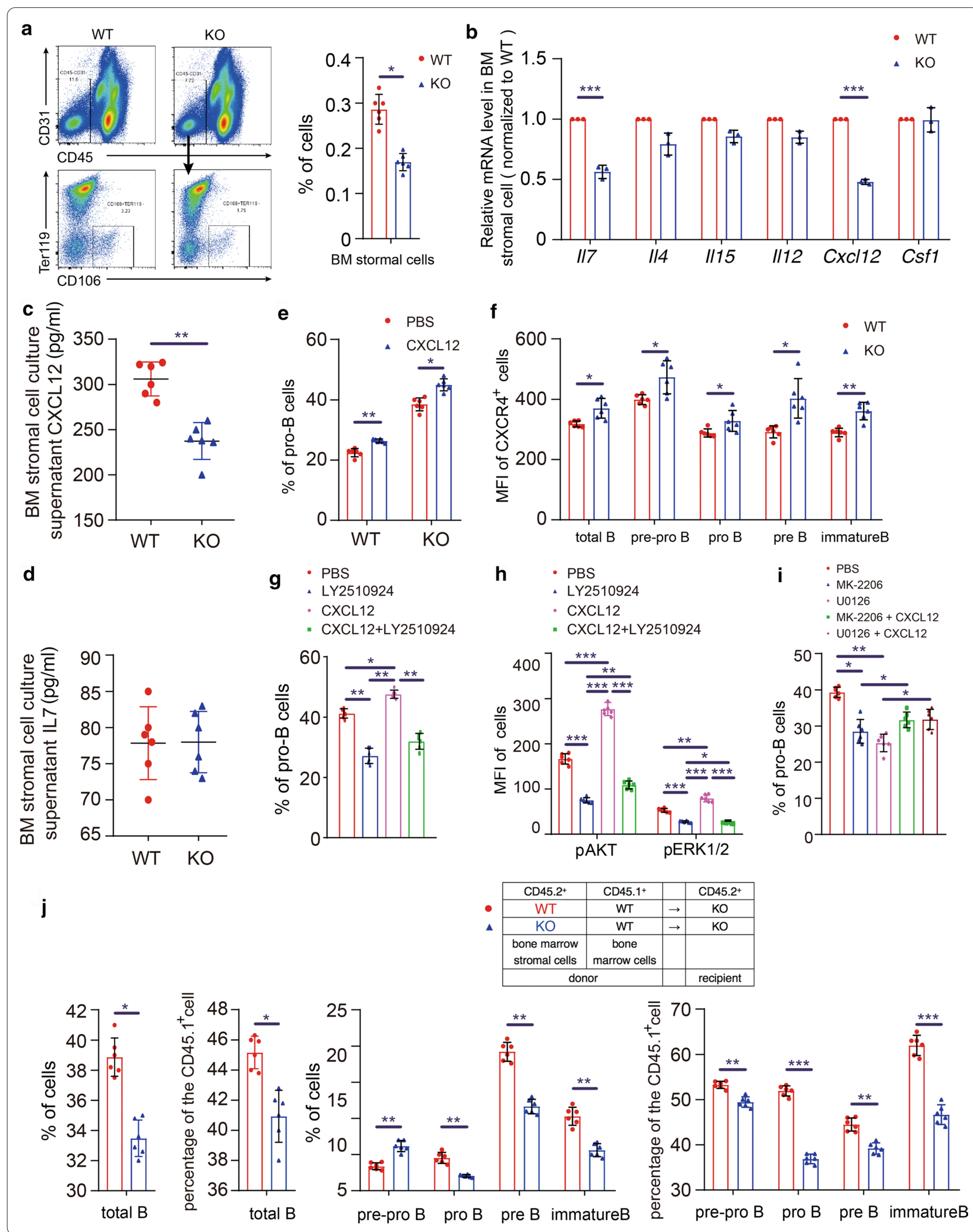
between them (Additional file 4 Fig. S3, Additional file 5 Fig. S4). Transcription factors Pu.1, Bcl11a, E2A, Ebf1, and Pax5 regulate the differentiation of common lymphoid progenitor (CLP) cells into B cell lineage [6]. Expression levels of *Pax5*, *Ebf1* and *Tcf3* in *Lf*$^{-/-}$ pre-pro-B and pro-B cells were higher than WT (Additional file 4 Fig. S3F, Additional file 5 Fig. S4E).

In another side, in vivo bone marrow transplantation experiment revealed that lactoferrin deficiency in bone marrow microenvironment retards the transition from pre-pro-B to pro-B stage (Fig. 1k). So, the dysregulation of early B cell development in lactoferrin-deficient mice is both cell autonomous and is associated with bone marrow microenvironment.

Proportion of stromal cells in *Lf*$^{-/-}$ bone marrow were decreased significantly comparing to WT (Fig. 2a). CXCL12 expressions were significantly lower in *Lf*$^{-/-}$ bone marrow stromal cells but not the IL-7 expressions (Fig. 2b–d). Lactoferrin can modulate *Cxcl12* expression in mouse stromal cell OP9 (Additional file 6 Fig. S5A, B); and CXCL12 protein promoted early B cell differentiation (Fig. 2e). CXCL12 modulate the differentiation of lymphocytes by binding to its receptor CXCR4 [7]. Expression of CXCR4 in B cells isolated from *Lf*$^{-/-}$ mice bone marrow was higher than WT (Fig. 2f, Additional file 6 Fig. S5C). CXCR4 inhibitor suppressed B cells differentiation, while CXCL12 can promote it (Fig. 2g). The CXCL12-CXCR4 axis transmits signals through

(See figure on next page.)

Fig. 2 Lactoferrin deficiency reduces the proportion of stromal cells in the bone marrow microenvironment and decreases CXCL12 expression to inhibit the early development of B cells. **a** Cells were isolated from the bone marrow of *Lf*$^{-/-}$ mice and WT littermates. Frequencies of bone marrow stromal cells (CD45$^-$CD31$^-$TER119$^-$CD106$^+$) were identified by flow cytometry. Representative data from 11 specimens each group are shown. **b** Expression levels of some factors in bone marrow stromal cells sorting from WT and *Lf*$^{-/-}$ mice were determined by RT-qPCR. n = 3. **c**, **d** ELISA was performed for CXCL12 and IL7 of the supernatant of bone marrow stromal cells (isolated from WT and *Lf*$^{-/-}$ mice). **e** In vitro differentiation experiment: bone marrow cells from *Lf*$^{-/-}$ or WT mice were added in 12-well plates, with CXCL12 recombinant protein (10 ng/ml), or PBS as control, for 9 days. The proportions of pro-B cells generated from the bone marrow cells were then determined by flow cytometric analysis. **f** Different stages of B cells were isolated from the WT and *Lf*$^{-/-}$ mouse bone marrow cells, and the surface expression of CXCR4 in each stage of B cells was determined by flow cytometric analysis. Each group has six mice. **g, h** In vitro differentiation experiment: bone marrow cells from *Lf*$^{-/-}$ mice were added in 12-well plates at 5 × 10^4 cells per well, with (1) PBS, or (2) LY2510924 (10 ng/ml), or (3) CXCL12 recombinant protein (10 ng/ml), or (4) LY2510924 (10 ng/ml) and CXCL12 recombinant protein (10 ng/ml), for 9 days. Each group has six specimens. The proportions of pro-B cells (**g**) and the phosphorylation levels of AKT and ERK in total B cells (**h**) generated from the bone marrow cells were then determined by flow cytometric analysis. **i** In vitro differentiation experiment: bone marrow cells from *Lf*$^{-/-}$ mice were added in 12-well plates at 5 × 10^4 cells per well, with (1) PBS, or (2) MK-2206 2HCL (10 ng/ml), or (3) U0126-EtOH (10 ng/ml), or (4) MK-2206 2HCL (10 ng/ml) and CXCL12 (10 ng/ml), or (5) U0126-EtOH (10 ng/ml) and CXCL12 (10 ng/ml), for 9 days. The proportions of pro-B cells generated from the bone marrow cells were then determined by flow cytometric analysis. **j** Bone marrow stromal cells transplantation experiment: bone marrow stromal cells from WT or *Lf*$^{-/-}$mice (CD45.2$^+$) were mixed with bone marrow cells from WT (CD45.1$^+$) mice at a ratio of 1:3, and then the mixed cells were transplanted into the irradiated lethal *Lf*$^{-/-}$ mice (CD45.2$^+$). After 6 weeks, the recipient mice were killed to prepare the bone marrow single-cell suspension, and the B cell proportion of each stage of B cell differentiation was analyzed by flow cytometry


a
WT
KO
CD31
CD45
Ter119
CD106
% of cells
WT
KO
BM stormal cells
b
Relative mRNA level in BM stromal cell (normalized to WT)
WT
KO
Il7
Il4
Il15
Il12
Cxcl12
Csf1
c
BM stromal cell culture supernatant CXCL12 (pg/ml)
WT
KO
d
BM stromal cell culture supernatant IL7 (pg/ml)
WT
KO
e
PBS
CXCL12
% of pro-B cells
WT
KO
f
WT
KO
MFI of CXCR4+ cells
total B
pre-pro B
pro B
pre B
immatureB
g
PBS
LY2510924
CXCL12
CXCL12+LY2510924
% of pro-B cells
h
PBS
LY2510924
CXCL12
CXCL12+LY2510924
MFI of cells
pAKT
pERK1/2
i
PBS
MK-2206
U0126
MK-2206 + CXCL12
U0126 + CXCL12
% of pro-B cells
j
CD45.2+ | CD45.1+ | | CD45.2+
WT | WT | → | KO
KO | WT | → | KO
bone marrow stromal cells | bone marrow cells
donor | | | recipient
% of cells
total B
percentage of the CD45.1+cell
total B
% of cells
pre-pro B
pro B
pre B
immatureB
percentage of the CD45.1+cell
pre-pro B
pro B
pre B
immatureB

MAPK and AKT signalings [8]. CXCR4 inhibitor reduced the phosphorylation levels of AKT and ERK, whereas CXCL12 increased them (Fig. 2h, Additional file 6 Fig. S5D). AKT or ERK inhibitors suppressed B cell differentiation, while CXCL12 can reverse their effects (Fig. 2i). So, *Lf*$^{-/-}$ mouse bone marrow stromal cells were not conducive to the early B cell development compared with WT controls (Fig. 2j).

Dysregulation of difference type B cells were observed in the spleen of *Lf*$^{-/-}$ mice comparing to WT (Additional file 7 Fig. S6A, B). Under the stimulation of TD and TI antigens, lactoferrin deficiency led to a decrease in proportion of total, follicular and T1 B cells (Additional file 7 Fig. S6C). The TNP-specific IgM and IgG2a, 2b responses were higher in *Lf*$^{-/-}$ mice (Additional file 7 Fig. S6D, E).

Systemic lupus erythematosus (SLE) is associated with a number of immunomodulatory abnormalities including B cell dysfunction [9]. In a pristane-induced SLE mouse model, the degree of injury to the glomerular filtration barrier, kidney damage, glomerular deposits of IgG antibodies, serum levels of IgM and IgG2b, amounts of anti-dsDNA total IgM and IgG in WT mice was lower than that in *Lf*$^{-/-}$ mice, and oral lactoferrin treatment alleviated the symptoms (Additional file 8 Fig S7). Lactoferrin plays a protective role in the SLE model.

In conclusion, this study demonstrates that lactoferrin is required for the early development of B cells in C57BL/6 mice by regulating the microenvironment of bone marrow stroma through CXCL12 release. *Lf*$^{-/-}$ mice had more severe symptoms in a SLE model, which can be alleviated by oral administration of lactoferrin, and that was related to the dysfunction of B cells induced by lactoferrin deficiency. Lactoferrin may be applied in preventative medicine or nutrition supplies for B cell-related diseases.

Abbreviations

CLP: Common lymphoid progenitor; CMP: Common myeloid progenitor; ELISA: Enzyme-linked immunosorbent assay; HSC: Hematopoietic stem cell; LF: Lactoferrin; SLE: Systemic lupus erythematosus; TI: T cell independent; TD: T cell dependent; WT: Wild type.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13045-021-01074-6.

Additional file 1: Methods.

Additional file 2: Fig. S1. Representative flow analysis diagrams for analysis of the different hematopoietic cells.

Additional file 3: Fig. S2. Representative flow analysis diagrams of in vivo bone marrow transplantation experiment.

Additional file 4: Fig. S3. Lactoferrin deficiency alters genes expression profile and key pathways in pre-pro-B cells.

Additional file 5: Fig. S4. Lactoferrin deficiency alters genes expression profile and key pathways in pro-B cells.

Additional file 6: Fig. S5. Effect of lactoferrin deficiency on Cxcl2 expression.

Additional file 7: Fig. S6. Lactoferrin deficiency affects the proportion of splenic B cells subclasses and antibody production in B cells.

Additional file 8: Fig. S7. Lactoferrin deficiency promotes the progression of SLE in mice.

Additional file 9: Table S1. Information on the antibodies used in FACS or mice experiments.

Additional file 10: Table S2. Antibodies information for ELISA.

Additional file 11: Table S3. Primer sequences used for qPCR.

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Authors' contributions

Conception and design were carried out by Wei and Ma. Wei, Liu, Wang, Xuemei Zhang, Ye, and Ma contributed to development of methodology. Xiang Zheng, Peng, Qin, Li, Xiaoyue Zhang, Wu, Wen, and Yan acquired the data. Analysis and interpretation of data were carried out by Wei, Wang, and Ma. Writing was done by Wei and Ma. Study supervision was done by Ma. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. The RNA-seq raw expression files and details have been deposited in NCBI GEO under accession number GSE163097.

Ethics approval and consent to participate

This study was approved by the ethical review committees of the Central South University.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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References

1. Brock JH. Lactoferrin–50 years on. Biochem Cell Biol. 2012;90:245–51.
2. He YM, Li X, Perego M, Nefedova Y, Kossenkov AV, Jensen EA, Kagan V, Liu YF, Fu SY, Ye QJ, et al. Transitory presence of myeloid-derived suppressor cells in neonates is critical for control of inflammation. Nat Med. 2018;24:224–31.
3. Wei L, Zhang X, Wang J, Ye Q, Zheng X, Peng Q, Zheng Y, Liu P, Zhang X, Li Z, et al. Lactoferrin deficiency induces a pro-metastatic tumor microenvironment through recruiting myeloid-derived suppressor cells in mice. Oncogene. 2020;39:122–35.
4. Ye Q, Zheng Y, Fan S, Qin Z, Li N, Tang A, Ai F, Zhang X, Bian Y, Dang W, et al. Lactoferrin deficiency promotes colitis-associated colorectal dysplasia in mice. PLoS ONE. 2014;9:e103298.
5. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005;102:15545–50.
6. Sigvardsson M. Molecular regulation of differentiation in early B-lymphocyte development. Int J Mol Sci. 2018;19:192.
7. Minami H, Nagaharu K, Nakamori Y, Ohishi K, Shimojo N, Kageyama Y, Matsumoto T, Sugimoto Y, Tawara I, Masuya M, et al. CXCL12-CXCR4 axis is required for contact-mediated human B Lymphoid and plasmacytoid dendritic cell differentiation but not T lymphoid generation. J Immunol. 2017;199:2343–55.
8. Cao Y, Hunter ZR, Liu X, Xu L, Yang G, Chen J, Patterson CJ, Tsakmaklis N, Kanan S, Rodig S, et al. The WHIM-like CXCR4(S338X) somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenstrom's Macroglobulinemia. Leukemia. 2015;29:169–76.
9. Liossis SC, Staveri C. B cell-based treatments in SLE: Past experience and current directions. Curr Rheumatol Rep. 2017;19:78.

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