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## ORIGINAL ARTICLE

# Hepcidin Induces M1 Macrophage Polarization in Monocytes or THP-1 Derived Macrophages

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## ABSTRACT

**Background:** Macrophage polarization plays a critical role in determining the inflammatory states. Hepcidin is a key negative regulator of iron homeostasis and functions. Although hepcidin has been shown to affect ferroportin expression in macrophages, whether it affects macrophage polarization is still largely unknown. **Objective:** To address whether hepcidin induces macrophage polarization. **Methods:** The expression of iNOS and CD206, and the ratio of IFN-γ vs IL-4 in THP-1 derived macrophages upon hepcidin stimulation were evaluated. Further detected was the percentage of $CD16^+$ M1, $CD23^+$ M1, $CD10^+$ M2 and $CCL22^+$ M2 cells in monocyte derived macrophages. **Results:** M1 associated molecules were increased in hepcidin-treated cells, yet M2 associated molecules were increased when hepcidin was neutralized. Concomitantly, we observed a significant increase in IRF3 phosphorylation in hepcidin-stimulated cells. However, STAT6 phosphorylation with hepcidin was neutralized. **Conclusion:** Hepcidin is able to induce macrophage polarization towards M1 type, and might be utilized as a potential M1 macrophage agonist in clinical practice.

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**Keywords: Hepcidin, Macrophage, Polarization**

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## INTRODUCTION

Macrophages play a key role as the front line of host defenses against pathogenic microorganisms. They can be polarized into two states depending on the type of secreted cytokines, i.e. classically activated macrophages (inflammatory or M1 macrophages) and alternatively activated macrophages (anti-inflammatory or M2 macrophages) (1). Proinflammatory M1 macrophages produce IFN-γ during antigen presentation and memory T cell activation, while alternatively activated M2 macrophages generate IL-4 and are involved in housekeeping functions, i.e. phagocytosis, tissue remodeling and immune suppression. M1 macrophages are characterized by a high capacity of antigen presentation, high inflammatory cytokine secretion, increased NO release, enhanced cytotoxic activity, and ability to induce Th1 immune response (1,2). Recently, owing to the abundance, broad distribution and powerful regulatory function of M1 macrophages, their induction and mobilization in tumor tissues has attracted tremendous research attention.

In multicellular organisms and nearly all microorganisms, as an essential trace element, iron catalyzes some enzymes in many redox reactions that are crucial for intermediary metabolism and energy production, such as the inflammatory response of macrophages following exposure to pathogens (3,4). Hepcidin is a major regulator of iron metabolism, also plays a role in inflammation, infection, and cancer progression (6,7). Once ligated to its receptor ferroportin, hepcidin causes internalization and degradation of the hepcidin-ferroportin complex, leading to reduced iron absorption and decreased iron export from macrophages (5). Under these conditions, iron is transferred from the circulation into storage, making it less available. Although hepcidin has been shown to effect iron retention in macrophages. It is still largely unknown whether it affect macrophages polarization. In this study, to explore how hepcidin polarizes macrophages, we evaluated the expression of iNOS and CD206, the ratio of IFN-γ vs IL-4 in THP-1 derived macrophages upon hepcidin stimulation. And we also detected the percentage of CD16+ M1, CD23+ M1, CD10+ M2 and CCL22+ M2 cells in monocyte derived macrophages.

## MATERIALS AND METHODS

**Hepcidin.** Human hepcidin peptides (DTHFPICIFCCGCCHRSKCGMCCKT) were synthesized at SciLight Biotechnology, LLC. The purity of hepcidin was >95% as confirmed by Mass spectrometry. Hepcidin was dissolved in PBS (pH 7.4) and filtered through a 0.22 μm syringe filter membrane.

**Cell Maintenance and Treatment.** The THP-1 cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified incubator (37°C, 5% CO2). THP1 cells ($2\times10^5$/ml) were differentiated into THP-1 derived macrophages using 200 nM PMA, phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 3d. Then the PMA-containing media was removed and changed to hepcidin (0 μM, 1 μM, 4 μM, 16 μM)-containing media and cells were kept culturing for another 24 h, followed by flow cytometry assay.

Human study was approved by the Luohe Medical College Research Ethics Committee and a written, informed consent was required from all subjets. The whole blood from healthy donors were treated with Ficoll Paque (GE healthcare) and centrifuged to isolate human peripheral blood mononuclear cells (PBMC), according to the product datasheet.

To induce monocyte derived macrophages (MDM), we plated $2\times10^6$ PBMC in 1 ml of RPMI 1640 media (Lonza) supplemented with 2 mmol/l L-glutamine (Gibco BRL) and 10% human AB serum (First Link Ltd. UK) into 24-well plates (Costar). Non-adherent cells in the medium were removed after 24 h, and the medium for the adherent cells was changed to RPMI with 10% heat-treated fetal bovine serum (FBS; Gibco). Then after 14d-culture, MDM concentration was approximately $2\times10^5$ MDM/ml. Macrophages derived from both THP-1 cells and monocytes were digested using pancreatic enzyme before flow cytometry assay.

**Flow Cytometry.** THP-1 derived macrophages were treated with hepcidin (4 μM) or anti-hepcidin (10 μg/ml) (Beijing Gegen Biotechnology, LLC) for 24 h before flow cytometry assay. Cells were incubated in 3% bovine serum albumin-PBS (Sigma, #B2064) containing anti-iNOS antibody (10 μg/ml) (Abcam, #ab15323) after cells fixed and permeabilized using FIX & PERM (Yeasen, #40402ES50&40403ES64), anti-CD206 antibody (10 μg/ml) (Abcam, #ab87099) or an isotype control (Abcam, #ab172730) at 4°C for 20 min, respectively. FcR blocking is necessary before antibody reactions. Cells were washed for three times with chilled PBS, and then incubated in PBS supplemented with 3% BSA, containing goat anti-rabbit IgG H&L conjugated with FITC (1:1000) (Abcam, #ab6717). After that, cells were washed for three times with chilled PBS, and fixed in 4% paraformaldehyde (Solarbio, #P1110). A MACSQuant® Analyzer (Miltenyi, Paris, France) was used for fluorescence intensity measure. The ratio of IFN-γ vs IL-4 in THP-1 derived macrophages was evaluated using intracellular cytokine staining. Briefly, cells were fixed and permeabilized with FIX & PERM, followed by incubation with FITC-conjugated anti-human IFN-γ (1 μg/ml) (Biolegend, #506504) or FITC-conjugated anti-human IL-4 (1 μg/ml) (Biolegend, #500807).

Blood macrophages were treated with hepcidin (4 μM), anti-hepcidin monoclonal antibody (Beijing Gegen Biotechnology, LLC) (10 μg/ml), or hepcidin (4 μM) plus LPS (100 ng/ml, Sigma, #L2630). The proportions of CD16+M1, CD23+M1, CD10+M2 and CCL22+M2 macrophages were then detected by incubation of macrophages with anti-CD16 (10 μg/ml) (Immunoway, #YM3090), anti-CD23 (10 μg/ml) (Immunoway, #YM0113), anti-CD10 (10 μg/ml) (Immunoway, #YM3072), or anti-CD22 (10 μg/ml) (Immunoway, #YM0113), or isotype control through flow cytometry assay, respectively. Goat anti-mouse IgG H&L (1:1000) conjugated with FITC (Abcam, #ab6785) was used as the secondary antibody to incubate cells at 4°C for 20 min.

**Immunoblot Assay.** Lysis buffer was purchased from Sigma (#04906837001). Cells were lysed for 30 min at 4°C in lysis buffer with protease inhibitor mixture (Sigma) and 1% Triton X-100 (Sigma) and then centrifuged at 20,000 g for 15 min at 4°C. Then the supernatants were mixed with 4X Laemmli buffer. Fifty micrograms of proteins were loaded for electrophoresis and transferred according to a standard protocol. Anti-IRF3 (10 μg/ml) (Immunoway, #YT5851), anti-pIRF3 (10 μg/ml) (Immunoway, #YP088) and anti-STAT6 (10 μg/ml) (Immunoway, #YT4454), anti-pSTAT6 (10 μg/ml) (Immunoway, #YP0255), β-actin (10 μg/ml) (Immunoway, #YM3121) antibody were used to detect corresponding signal pathways. The chemiluminescence was analyzed with chemiluminescent detection kit (GE Healthcare, #RPN2105).

**ELISA.** The cytokine (IL-4 and IFN-γ) concentration in the supernatants of THP-1 derived macrophages with or without hepcidin treatment were evaluated using Multi Analyte ELISA Array kit (Qiagen, MEH-004A and MEH-009A), according to the product datasheet.

**Statistical Analysis.** All the results were the average of three independent assays and were expressed as the mean ± SD. Paired Student *t test* was performed for the statistical analysis, and $p<0.05$ was considered statistical significance.

## RESULTS

**Hepcidin up-regulates the M1 polarization of THP-1 derived macrophages.** Firstly, we investigated the effect of hepcidin on THP-1 derived macrophages. PMA was used to facilitate THP-1 differentiation. THP-1 derived macrophages were treated with 0 μM, 1 μM, 4 μM, or 16 μM concentration of hepcidin. Expression of inducible nitric oxide synthase (iNOS), an M1 type marker, and CD206, an M2 type marker, were determined using flow cytometry. Hepcidin was utilized to induce iNOS, and hepcidin neutralizing antibody was employed to block the function of hepcidin. It was observed that hepcidin increased the expression of iNOS in THP-1 derived macrophages in a dose dependent manner (Figure 1A). Four μM hepcidin, a concentration inducing appropriate reactivity, was selected to do the following assay. In this study, four groups were created, namely hepcidin (+), anti-Hepcidin (-) (Hepcidin group), hepcidin (-), anti-Hepcidin (+) (anti-Hepcidin group), hepcidin (-), anti-Hepcidin (-) (medium control group), and hepcidin (+), anti-Hepcidin (+) (hepcidin and anti-Hepcidin group). As shown in Figure 1B, compared with other three groups, hepcidin group had significantly higher iNOS expressions, and anti-hepcidin group had the highest CD206 expression ($p<0.01$). Representative histograms of iNOS and CD206 expression after hepcidin and its neutralizing antibody treatment are also shown. Further tested was whether hepcidin impacted the expression of IFN-γ and IL-4 in THP-1 derived macrophages. IFN-γ, an M1 type event, and IL-4, an M2 type event, were determined using flow cytometry and cytokines ELISA assay. Hepcidin was used to induce IFN-γ and IL-4 expressions, which were increased in a dose dependent manner in THP-1 derived macrophages in both cells and supernatants (Figures 2A, 2B and 2C). Representative histograms of IFN-γ and IL-4 expression with or without hepcidin treatment are shown on the right panel of Figures 2A and 2B. The ratio of IFN-γ vs IL-4 was also compared. As shown in Figure 2D, although the expression of IL-4 increased with hepcidin stimulation, the ratio of IFN-γ vs IL-4 was significantly higher in different hepcidin-treated groups compared with the medium control group concerning both flow cytometry assay and cytokines ELISA assay ($p<0.01$). These results indicated that hepcidin was able to induce the differentiation of THP-1 derived macrophages into M1 macrophages, while hepcidin neutralizing antibody could enhance the M2 macrophage phenotypes of THP-1 derived macrophages.

**Hepcidin increases the abundance of M1 monocyte derived macrophages in PBMC.** To test the effect of hepcidin on blood macrophages, PBMCs were seeded into 24-well plates. 10% human AB serum was used to treat the monocyte in PBMC because this kind of serum contained some cytokines to promote macrophages differentiation (8). After 24-h incubation and aspiration of floating cells, MDMs were kept for further tests. Hepcidin, hepcidin neutralizing antibody, and hepcidin plus LPS were used to treat MDMs. After that, four macrophages markers, i.e. CD16 and CD23 (M1 type markers), CD10 and CCL22 (M2 type markers) were detected in these MDMs. LPS acts as the prototypical

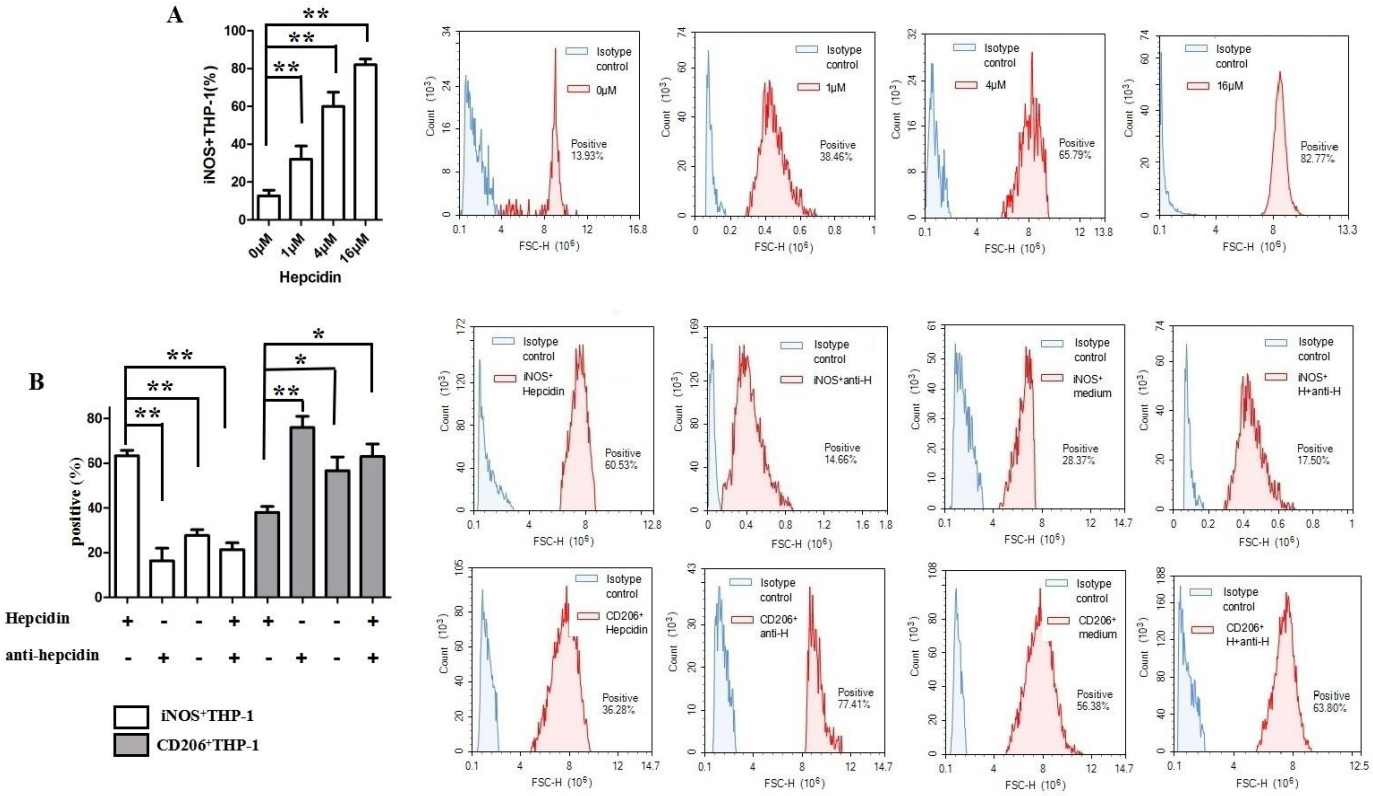


**Figure 1. Hepcidin increased iNOS expression and decreased CD206 expression in THP-1 derived macrophages. A** Left panel, the proportion of iNOS$^+$THP-1 derived macrophages after different concentration of hepcidin treatment. The data were summarized from three independent assays. The percentage of iNOS$^+$THP-1 derived macrophages increased significantly in a dose dependent manner ($p<0.01$). Right panel, representative histograms of iNOS expression in different concentration of hepcidin. **B** Left panel, the proportions of iNOS$^+$M1 and CD206$^+$M2 in THP-1 derived macrophages after hepcidin or neutralizing antibody treatment. Hepcidin significantly up-regulated the percentage of iNOS$^+$M1 macrophages ($p<0.01$), while significantly down-regulated the percentage of CD206$^+$M2 macrophages ($p<0.01$). Right panel, representative histograms of iNOS and CD206 expression in THP-1 derived macrophages. *, $p<0.05$. **, $p<0.01$.

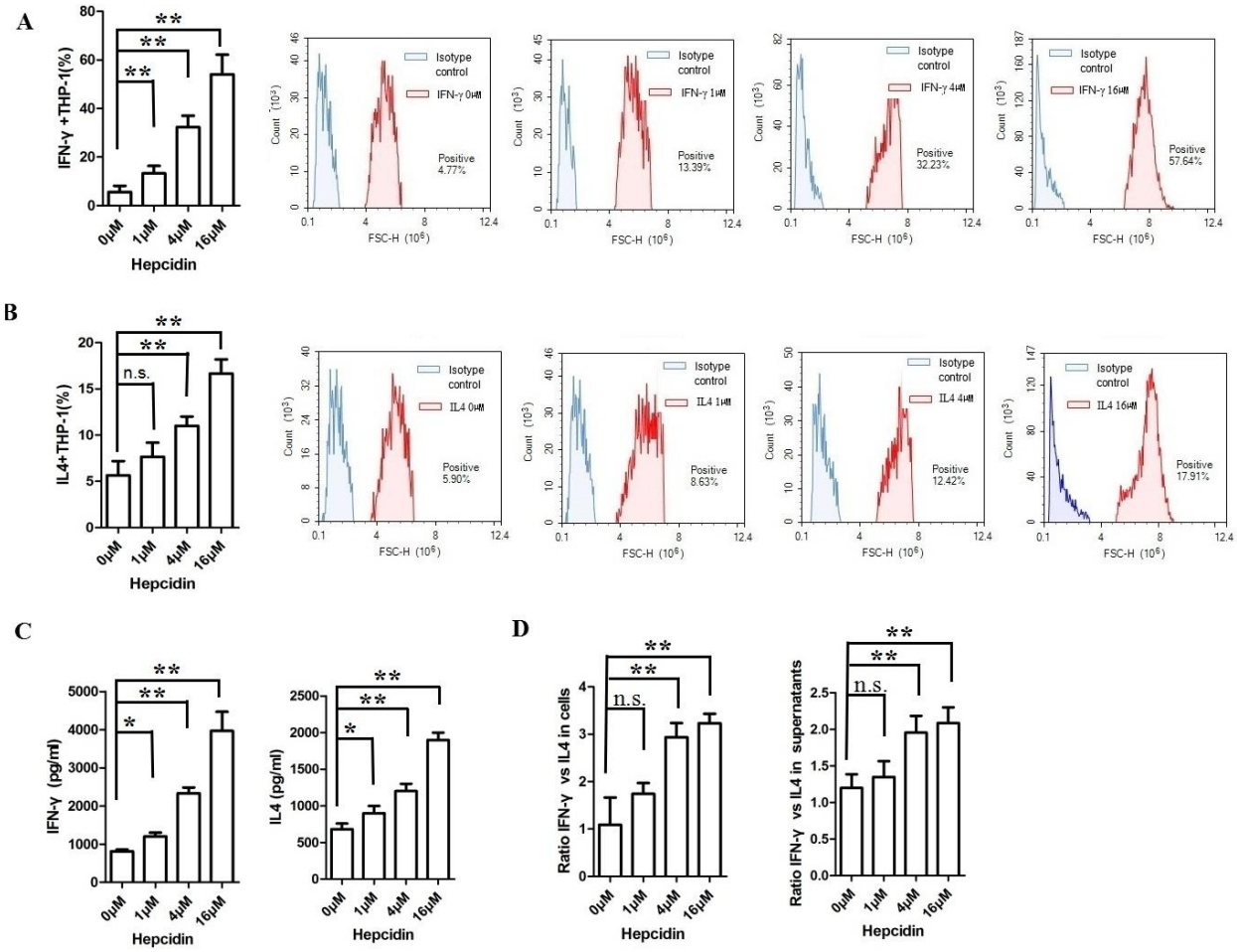


**Figure 2. The ratio of IFN-γ vs IL-4 in hepcidin-treated group was higher than medium control group. A** Left panel, IFN-γ expression in THP-1 derived macrophages on different concentration of hepcidin treatment. Right panel, representative flow cytometry histograms of IFN-γ expression in THP-1 derived macrophages. **B** Left panel, IL-4 expression in THP-1 derived macrophages on different concentration of hepcidin treatment. Right panel, representative flow cytometry histograms of IL-4 expression in THP-1 derived macrophages. Hepcidin significantly increased the production of IL-4 and IFN-γ in a dose dependent manner ($p<0.01$). However, 1 μM hepcidin had no effect on IL-4 expression. The percentage of IFN-γ was higher than IL-4 in different hepcidin treatment groups. The data were summarized from three independent assays. **C** The concentration of IFN-γ (left panel) and IL-4 (right panel) in the supernatants of THP-1 derived macrophages with or without hepcidin treatment. IL-4 and IFN-γ expression increased significantly in a dose dependent manner. The data were summarized from three independent assays. **D** Statistics for the ratio of IFN-γ vs IL-4 both in cells (left panel) and in supernatants (right panel). The ratio of IFN-γ vs IL-4 were significantly higher on 4 μM and 16 μM hepcidin treatment compared with no hepcidin added group. *, $p<0.05$. **, $p<0.01$, n.s., no significant.

endotoxin to promote cells such asmonocytes, dendritic cells, macrophages and B cells to secrete nitric oxide, eicosanoids and pro-inflammatory cytokines (9). We wonder if LPS had synergy effect with hepcidin. As shown in Figure 3, in comparison with control group, either hepcidin alone or hepcidin plus LPS profoundly increased the proportion of $CD16^+$ M1 and $CD23^+$ M1 macrophages. With regard to $CD10^+$ M2 and $CCL22^+$ M2 macrophages, hepcidin neutralizing antibody robustly increased their abundance, whereas hepcidin with or without LPS significantly reduced the abundance of $CD10^+$ M2 and $CCL22^+$ M2 macrophages. LPS had synergy effect with hepcidin to reduce the proportion of $CD10^+$ M2 and $CCL22^+$ M2 macrophages. Interestingly, in comparison with hepcidin alone, the presence of LPS down-regulated the proportion of $CD16^+$ M1 macrophages but up-regulated the proportion of $CD23^+$ M1 macrophages. It is indicated that there is different signal pathway between hepcidin and LPS. Representative histograms of CD16, CD23, CD10 or CCL22 expression after hepcidin, hepcidin plus LPS and its neutralizing antibody treatment were also shown.

**Hepcidin induces M1 Polarization through inhibition of STAT6 signaling and activation of IRF3 signaling.** To ascertain the signal pathways underlying hepcidin-induced changes in monocyte derived macrophages, phosphorylation of two transcription factors was assessed. Interferon regulatory factor 3 (IRF3) and the phosphorylation of IRF3, which is related to IFN-γ expression, and signal transducer and activator of transcription 6 (STAT6) and the phosphorylation of STAT6, which is related to IL-4 expression, were detected. As shown in Figure 4, the protein levels of IRF3 and STAT6 were roughly comparable in each group, suggesting that these treatments did not alter the expression of these two factors apparently. Whereas hepcidin up-regulated the phosphorylation of IRF3 but down-regulated the phosphorylation of STAT6 as compared with control and hepcidin neutralizing antibody treatment group ($p<0.01$). Hepcidin neutralizing antibody decreased IRF3 phorsphorylation but increased STAT6 phosphorylation in comparison with hepcidin treatment group ($p<0.01$). This result suggests that IRF3 and STAT6 signaling are involved in hepcidin-induced polarization in macrophages. The IRF3 signaling is related to M1 polarization and the STAT6 signaling is related to M2 polarization, respectively.

## DISCUSSION

In this report we studied the effect of hepcidin on macrophages polarization. We found that hepcidin induced M1 polarization of macrophages derived from both THP-1 cells and monocytes by affecting IRF3 phosphorylation, and the polarization is associated with elevated expression of iNOS, CD16, CD23, and increased secretion of IFN-γ. LPS could enhance some hepcidin-induced M1 and M2 type markers expression. M1 polarization is induced by Toll-like receptor (TLR) ligands and IFN-γ. M2 polarization can be divided into subtypes such as M2a, M2b and M2c, which were induced by IL-4/IL-13, immune complex, and IL-10 or TGF-β, respectively (10,11). This phenomenon mirrors the Th1/Th2 polarization in T cell response (12-14). So IFN-γ and IL-4 secretion could reflect the macrophagess polarization status. We also analyzed IRF3 and STAT6 signal pathways which have been proved essential for IFN-γ and IL-4 signaling. IL-4 induces

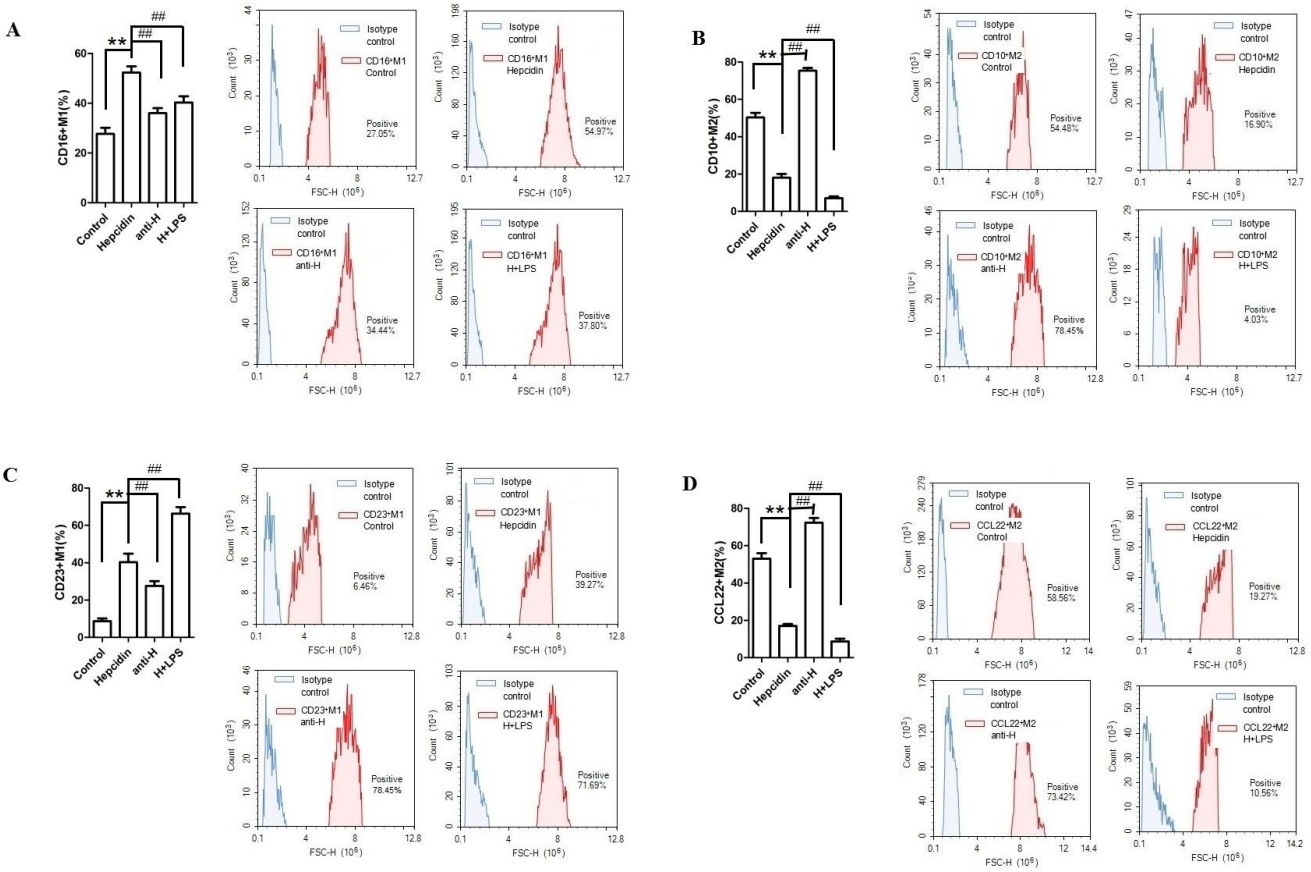


**Figure 3. Hepcidin induces M1 polarization in monocyte derived macrophages. A** Left panel, the percentage of CD16$^+$M1 monocyte derived macrophages under different treatment. Right panel, representative flow cytometry histograms of CD16$^+$M1 monocyte derived macrophages proportion under different treatment. **B** Left panel, the percentage of CD10$^+$M1 monocyte derived macrophages under different treatment. Right panel, representative flow cytometry histograms of CD10$^+$M1 monocyte derived macrophages proportion under different treatment. **C** Left panel, the percentage of CD23$^+$M2 monocyte derived macrophages under different treatment. Right panel, representative flow cytometry histograms of CD23$^+$M2 monocyte derived macrophages proportion under different treatment. **D** Left panel, the percentage of CCL22$^+$M2 monocyte derived macrophages under different treatment. Right panel, representative flow cytometry histograms of CCL22$^+$M2 monocyte derived macrophages proportion under different treatment. The data were summarized from three independent assays. Control group was no hepcidin added, hepcidin group was 4μM hepcidin added, anti-H group was 10 μg/ml hepcidin neutralizing antibody added, and H+LPS group was 4 μM hepcidin plus 100 ng/ml LPS added. In comparison with control group, hepcidin treatment group had significantly changed the expression of these four markers ($p<0.01$). Furthermore, hepcidin treatment group had significantly changed the expression of these four markers in comparison with anti-H group ($p<0.01$). **, $p<0.01$. ##, $p<0.01$. LPS enhanced the down-regulation of hepcidin with CD10$^+$ and CCL22$^+$M2 markers, while LPS had contradictory effect on up-regulation of hepcidin with CD16$^+$ and CD23$^+$M1 markers.

the serine phosphorylation of STAT6 and STAT6-bound enhancers repress macrophage transcription, and subsequently affects macrophage inflammatory response indued by LPS. It suggests that during M2 polarization also occurs direct transcriptional repression (15,16). Iron metabolism has been characterized in macrophages-mediated inflammation (17-19). Hepcidin has 25 amino acids and is a key regulator of iron metabolism. Our results suggest that hepcidin induces M1 macrophages polarization. However, in other study, it was shown iron reduced M1 polarization of RAW264.7 macrophages (20).

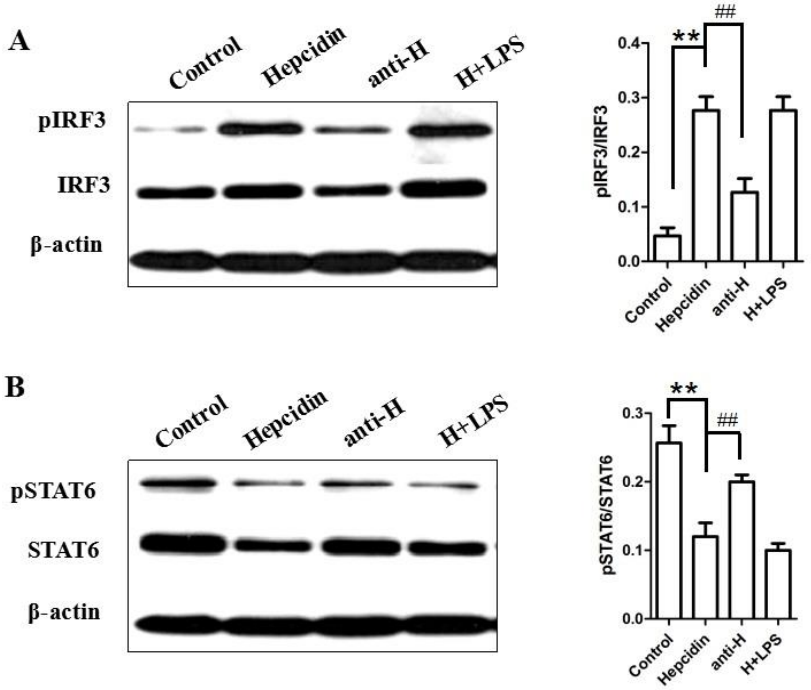


**Figure 4. Phosphorylation of IRF3 and STAT6 in monocyte derived macrophages. A** Left panel: representative images of immunoblot assay for total IRF3 and phosphorylated IRF3 in different treatment groups. Right panel: Statistics for the ratio of pIRF3 vs IRF3. **B** Left panel: representative images of immunoblot assay for total STAT6 and phosphorylated pSTAT6 in different treatment groups. Right panel: Statistics for the ratio of pSTAT6 vs STAT6. Densitometric analysis was performed using pooled data from three such experiments. **, $p<0.01$. ##, $p<0.01$.

This discrepancy may be caused by factors such as differential simulation, iron concentration, cell condition. In that study (20), iron was added directly into RAW264.7 macrophages, the increased concentration of iron may induce more complicated responses, however in our study, hepcidin was added into macrophages without changing the iron concentration in the medium, so the activated ferroportin receptor signaling is more specific. In summary, macrophages polarization plasticity has important therapeutic implications, and hepcidin is a potential therapeutic agent for macrophages-centered treatment for various diseases.

## ACKNOWLEDGEMENTS

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