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Hepcidin mediated iron homeostasis as immune regulator in visceral leishmaniasis patients

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Abstract

Aim: Iron is key ingredient for immunosurveillance and host-pathogen interaction. Intracellular pathogen steals the iron from the host, but how parasite orchestrates iron acquisition and affects immune responses remains controversial. We aimed to study the iron homeostasis in visceral leishmaniasis (VL) and its influence on immune machinery.

Methods and Results: This study was performed on purified monocytes and T-cells, peripheral blood mononuclear cells and splenic aspirates for transcriptional analyses of iron homeostasis (hepcidin, DMT-1, transferrin receptor, ferroportin) and immune modulations (IFN- γ , HLA-DR, IL-10, iNOS, IL-6). Serum/plasma were used for determination of iron, total/transferrin iron binding capacity and anti-leishmania antibody titres in cases.

We report that VL induced perturbation in iron homeostasis may cause immune dysfunctions. VL cases had decreased iron uptake by transferrin dependent and independent routes while elevated hepcidin, degraded sole iron exporter ferroportin. Therefore, it appears that perturbation in iron homeostasis has essential role in HLA-DR mediated antigen presentation and innate armory by downregulating iNOS as well as altering IFN- γ , IL-6, IL-10 profiles.

Conclusion: The iron homeostasis by hepcidin can serve as one of the crucial determinants for regulating immune cell signaling therefore, targeting iron metabolism, specifically hepcidin alone or in combination with agonists can serve to clear infection.

Summary:

Iron regulates the immune responses in human *L. donovani* infection

Keywords

Visceral leishmaniasis; iron homeostasis; anemia; hepcidin; antigen presentation; pro-inflammatory cytokines; nitric oxide

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Introduction

Iron has been known as key molecule for the immunosurveillance and decisive factor for host-pathogen interaction. *Leishmania*, being an intracellular parasite exploits host's iron as major nutritional resource for its survival and virulency, leading to the clinical condition called anemia, key manifestation of visceral leishmaniasis (VL). The relationship between iron deficiency and susceptibility to infectious disease has been known with the essential role of iron in the development of immune system ¹. *Leishmania donovani* infection causes fever, anemia, splenomegaly, pancytopenia and hepatomegaly leading to fatal VL by entering the host macrophages, where it proliferates, multiplies and bursts the host cell to infect neighboring immune cells. It acquires iron from host for its intracellular growth and subverts oxidative assault imposed by the host ². Moreover, intracellular level of iron affects their drug-resistance abilities therefore, acquisition of iron is important for the survival and virulency of the parasite.

On the other hand, host employs iron as key regulator for building and/or boosting immune responses against pathogens. The defense against intracellular pathogen relies on immune recognition by mononuclear phagocytic system and its collaboration with T-cells, raising TH1-dependent pro-inflammatory responses for eliminating the pathogen. Iron has been key regulator for boosting this cell-mediated immune response by driving proliferation, metabolic fitness and microbicidal mechanisms ^{3, 4} in different immune cells. The immune cells have to protect their iron from the intracellular pathogens to maintain their functions, since iron constitutes key ingredient in the protection against invading pathogen by serving as cofactor for the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Moreover, macrophages play crucial role in body iron homeostasis by recycling and redirecting it for erythropoiesis⁵. Therefore, cellular iron levels must be carefully regulated by *Leishmania*, because apart from being the precursor for iron dependent enzymes and Fe-S cluster, iron also serves for free radical production as discussed above, to limit the availability of iron to pathogens. This involves the host mononuclear system which chelates iron making it inaccessible to pathogens. In order to survive and replicate, *Leishmania* is equipped with iron acquiring mechanisms⁶ that harnesses iron sources from the macrophages, suggesting for the essence of iron for pathogenicity and survival of parasite leading to anemia of chronic disease(ACD).

Lymphocytes also possess limited ability to sequester iron, T-cells are more sensitive to changes in iron homeostasis than B-cells ⁷. Reduction in iron availability interferes with lymphocyte proliferation, activation and cytokine production thereby dampening the immune effector functions. The major iron acquisition routes in leukocytes are *via*. transferrin mediated endocytosis by transferrin receptor, TfR ⁸, iron bound to divalent metal transporter1 (DMT1 or Nramp2) ⁹ and phagocytosis of senescent red blood cells while iron efflux is mediated solely by ferroportin (FPN) ¹⁰, which in turn is inversely regulated by the level of hepcidin (HPN) in infections and upon iron overload ^{11,12} by inhibiting the antigen presentation and microbicidal activity in the form of hampered RNS production by iNOS. Also, the close interconnection between iron and immunity is governed by different cytokines which alter the cellular iron metabolism ¹³. This tendency of host immune system to withhold iron from intracellular pathogens and limit their proliferation, in addition, to

consolidation of its own immune effector pathways, this has been termed as nutritional immunity. Therefore, preventing the access of iron to pathogen is extremely important for immune cell functionality and homeostasis.

Cellular stress by heme oxygenase-1, during *L.chagasi* infection has also been shown to be responsible for inflammatory imbalance¹⁴ and inhibition of lymphoproliferative responses¹⁵ thereby conferring disease susceptibility. Therefore, iron serves as double-edged sword-as a nutrient for pathogen and antimicrobial defense, becoming an important parameter in context of macrophage-residing *Leishmania*, determining the disease fate and how this balance is regulated by HPN has been a mystery in VL infection. HPN, iron regulatory capacity and antimicrobial activity utilizes potentially unexplored iron regulatory pathway. It is speculated that alterations in iron regulatory pathways can have potential implication in host defense by limiting the availability of iron to the pathogen. Apart from being the weapon of innate system HPN has also been documented to be expressed on T-cells, required for the activation and proliferation of these immune cells¹⁶. Despite of the proven role of iron homeostasis, the concept of interconnection between iron and disease susceptibility still remains controversial in VL.

So far, there has been no evidence in any clinical setup for the role of iron and its associated transporters in the VL pathogenesis. We aimed for prospective study of effect of VL associated anemia and iron homeostasis on cellular immunity during VL, ultimately determining pathogenicity of disease. These findings documents the role of iron acquisition machinery in VL cases as the key player in determining parasite mediated immune modulations with direct effects on cytokine-mediated immune effector functions for its pathogenesis therefore, providing us the key to explore the iron homeostasis pathways for therapeutic benefit and improving our understanding on host-pathogen interactions in infectious diseases.

Materials and methods

We had performed the study on VL patients recruited to Kala-Azar Medical Research Centre (KAMRC), Muzaffarpur, Bihar, India for their diagnosis and further treatment. The diagnosis was done by observation of typical symptoms with blood cell reports further confirmed with detection of antibodies in the serum with recombinant K39 and/or amastigotes in the splenic aspirates by microscopy. The study was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University and all subjects provided written informed consent. Aggregate clinical data has been presented in Table 1, obtained from complete blood count of confirmed VL cases upon admission to KAMRC. Iron and total iron binding capacity (TIBC) was accessed via. ferrozine method based upon spectrophotometric evaluation of violet coloured complex as reported by Siedel *et al.*¹⁷.

Direct agglutination test (DAT) was performed with the serum samples as described elsewhere¹⁸, using a freeze dried version of fixed, trypsin treated and stained promastigotes of *L. donovani* prepared in ITM-Antwerp, Belgium¹⁹. For the purpose of analysis, DAT seropositivity was defined as a DAT end titer of 1:3200 or above²⁰. Splenic aspirates were taken as part of routine diagnostic procedure at the KAMRC, Muzaffarpur, Bihar State,

India. Pre- and post-treated cases were subjected to collection of splenic aspirates in RLT buffer (Qiagen GmbH, Hilden, Germany). Venous blood (heparinized) was collected from patients and endemic healthy subjects (EHC) for PBMC isolation by ficoll based density gradient method, by layering the blood onto the ficol. A portion of PBMCs were washed and resuspended in MACS buffer (0.5% BSA, 2mM EDTA in 1X PBS). Monocytes and T-cells were enriched by positive selection using CD14 and CD3 microbead kits respectively, with a magnetic MS column according to the protocol described by the manufacturer (Miltenyi Biotec).

Total RNA was isolated using RNeasy tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Sample quality and integrity was assessed by ND-2000 spectrophotometer (Thermo Fischer Scientific Wilmington, DE, USA) and agarose (Sigma Aldrich Chemicals, St Louis, USA) gel electrophoresis. 1000ng of RNA was reverse transcribed using the high capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions. 7500 REAL TIME PCR platform (ABI, Foster City CA, USA) was used with TaqMan assay for following genes- HLA-DRB1(Hs04192464_mH), IL-6 (Hs00985641_m1), IL-10(Hs00961622_m1) and IFN- γ (Hs99999041_m1) and SYBR Green based gene expression assays were performed for DMT1, FPN, HPN as described previously ²¹, TfR1²² and iNOS (forward 5'-TCATTCTGCTGCTTGCTGAG-3' and reverse 5'-CAACAATGTGGAGAAAGCCC-3'). 25ng of cDNA used for experiment as 5ul of cDNA (5ng/ μ l), 1 μ l of primer/probe 4 μ l of MilliQ and 10 μ l of TaqMAN/SYBR green master mix (Applied Biosystems, Foster City, CA, USA) with amplification in 7500 Real-Time PCR system (ABI). The thermal cycling condition included an initial incubation at 50°C for 2 minutes, followed by a 10 minutes, denaturation at 95°C and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute each. The study was performed on 63 samples including the paired pre-(D-0)/ post-treatment(D-Dis) or unpaired pre/post-treated splenic aspirates (n=12), PBMCs (n=28), purified monocytes, T-cells and endemic healthy controls (n=20) with appropriate no RT and no template controls included in each plate. All samples were run in duplicate. Comparative delta Ct was performed using endogenous control 18S rRNA (4333760F) for Taqman assay and GAPDH ²¹ for SYBR green based assays respectively. The results obtained were analyzed by 7500 ABI SDS software v.2.0.1 and Graph pad prism 5 (version 5.00 for Windows, Graph Pad Software, San Diego California USA). The significance of differences between groups was determined using student's t-test or, if samples failed to normality distribution parameters, non-parametric Mann-Whitney test was used; where applicable paired t-test or Wilcoxon-Signed rank test was used.

Results

VL associated anemia also called as ACD, has been a common manifestation of VL which resolves after therapy. Iron is essential for build-up of blood cells and insufficient iron during ACD can perturb the iron homeostasis. Considering 93 VL cases admitted for the study with anemia and leucopenia (Figure 1a) as the key manifestations, we sought to establish relationship between leukocyte count and hemoglobin levels in VL cases who were not receiving any antileishmanial treatment at the time of study. There was significant difference in the levels of hemoglobin in VL cases pre (D-0) and thirty days post (D-Dis) treatment

suggesting for the disease associated anemia during chronic infection (Figure 1a). Similarly, significantly high level of leukocyte count per microlitre blood was observed upon post-treatment (Figure 1b). This, indeed, raised concern to study correlation between two parameters during chronic *Leishmania* infection. We observed positive correlation between leukocyte count and hemoglobin levels ($p=0.0060$ and $r=0.2991$) (Figure 1c). This may indicate for the correlation between severities of symptoms, however, no differences in the serum TIBC between the infected and healthy controls (EC) (Figure 1d) were observed. To further delineate the relationship between hemoglobin and immunity, the parasite specific antibody titres and hemoglobin was determined. As shown in figure 1e, statistically significant inverse correlation was found between the two parameters ($p\text{-value}=0.0042$ and $r=0.6306$), indicated for the decreased hemoglobin levels with increased parasite specific antibody responses. This was further validated in terms of inverse correlation between the spleen size of pre-treated (D-0) cases with the hemoglobin levels (Figure 1f), indicated for the lower hemoglobin levels with the severe infection seen in the form of high parasite-specific antibody titres as well as the spleen size. Since iron is the key component for the hemoglobin production, these findings ignited the concern for studying the iron homeostasis in VL pathogenesis. However, during intracellular infections, iron withholding is reinforced by the host, thus, genes involved in iron acquisition has an important role in determining the disease pathogenesis and host immune responses²³. The investigation of transcriptional profile of iron uptake and homeostatic mediators, HPN and TfR1 revealed for approximately similar trends in spleen and PBMCs. However, spleen remains more sensitive to even minor fluctuations in the iron homeostatic pathways as compared to PBMCs as observed from significant differences between pre- and post-treated cases. We observed decreased expression of TfR1 for transferrin-dependent uptake ($p\text{-value}=0.1482$ and 0.0131 in PBMC and spleen respectively) during infection. However, the pre-treated cases had compromised expression as compared to healthy controls in PBMCs ($p\text{-value}=0.0011$) as shown (Figure 2a). However, the relative expression in PBMCs was further reflected in the monocyte/macrophage population with elevated expression in healthy controls as compared to pre-treated cases while treatment duration. Furthermore, decreased TfR1 expression in post-treated monocytes/macrophages compared to pre-treated cases was quite significant while in the PBMC transcript levels were unaltered during course of treatment. When relative expression pattern for TfR1 was assessed between the purified cell subsets, elevated TfR1 expression was found in T-cells at pre-treatment stage compared to the monocytes/macrophages. This could be attributed for the differentiation stage of monocytes/macrophages as well as their infection status rather than proliferation; therefore, they do not solely depend on TfR1 mediated iron uptake. Interestingly, the altered levels of TfR1 expression were accompanied with no significant alteration in transferrin-binding capacities (TIBC) between the controls and cases (Figure 1d).

Likewise, the DMT-1 dependent route of iron uptake was also unaltered during the course of treatment (pre-and post-treatment) in spleen and PBMCs (Figure 2b) without any significant differences in the iron uptake machinery in PBMCs at different stages of treatment, while diminished expression was observed during the entire course of disease (i.e. pre- and post-treated) as compared to healthy controls ($p\text{-value}<0.0001$) (Figure 2a) signifying the diminished iron uptake in VL infection which persisted even after completion of treatment.

Similarly, monocytes/macrophages reflected the PBMCs trend, in terms of decreased transcript levels in pre- and post-treated subjects compared to healthy controls, with no significant differences in DMT-1 expression in PBMCs and spleen; while, the T-cells expressed elevated levels of DMT-1 than monocytes/macrophages in pre-treatment stage. Therefore, *L. donovani* infection caused significantly reduced cellular iron uptake when compared to endemic healthy controls in terms of TfR1 and DMT1, indeed, the cellular machinery employs strategies to limit the availability of iron for parasite proliferation. This raised the concern to further unveil the iron homeostasis in immune cells in terms of iron-regulatory hormone HPN and FPN transcripts, to provide evidences for parasite adaptive strategy against the cellular machinery and utilization of its sources. Elevated transcript levels of HPN were observed in cases as compared to healthy controls, without any significant changes in the transcripts during the course of treatment in PBMCs (Figure 2c). Conversely, spleen had decreased expression of HPN in post-treated cases; however, PBMCs had higher HPN transcripts than spleen. Monocytes/macrophages had higher transcripts in pre-treated compared to post-treated and healthy controls, replicating the PBMC dataset. When HPN levels were compared in each cell subsets monocytes/macrophages had elevated transcripts than the T-cells, reflecting the macrophage iron retention armory for exploitation by the parasite for its own benefit. This is the first report for the analysis of leukocyte-derived HPN and its regulators in human VL; we believe that these differences in the expression are of relevance.

Determination of the transcript levels of FPN showed similar trend in PBMC and spleen without any changes in either pre- and/or post-treated subjects or pre-treated versus healthy controls and post-treatment (Figure 2d). However, PBMCs had lower transcripts as compared to the spleen, therefore, retaining iron within the cell and its utilization by the parasite causes perturbation in cellular iron pool. HPN-mediated iron retention and immune inhibitory effects of iron has been known but little is known about its impact on immune functions. We assessed immune activation marker HLA-DR in these cases at pre-/post-treatment as compared to controls, which was significantly reduced in VL cases as compared to controls, without any significant changes in the in pre- and post-treatment stages (Figure 3a). It could be assumed as a consequence of iron-induced suppression of the immune stimulatory effects of IFN- γ on antigen presentation by HLA-DR.

iNOS was another heme-containing effector molecule, whose expression was compromised during infection as compared to healthy controls which upon treatment regained similar state as healthy controls (Figure 3b). While no significant differences were observed in spleen in the pre- and post-treated cases, possibly due to residual infection in the spleen upon completion of treatment tenure. PBMCs had higher transcriptional levels in post-treated cases as compared to pre-treated group. However, no significant differences were observed in transcripts of iNOS in PBMCs and spleen of pre-treated cases, signifying the similar extent of compromised effector functions in spleen and PBMCs.

Further HPN-production and immune modulatory effects are known to be directly controlled by pro- and anti-inflammatory cytokines. We studied the involvement of these cytokines, in terms of their transcripts - IFN- γ , IL-6 and IL-10. IFN- γ transcript levels in pre- and post-treatment (p-value<0.0001 and p-value=0.0001 for PBMC and spleen respectively) (Figure

4a), while higher expression was detected in PBMC of cases as compared to healthy controls that had gone down upon treatment, gradually attaining transcript levels similar to healthy controls. Also, PBMCs and spleen had almost similar levels of IFN- γ transcripts, suggestive of similar levels of IFN- γ irrespective of the site of infection. The effect of iron on pro-inflammatory responses was further validated by transcriptional analysis of another inflammatory marker IL-6, which was also altered during active VL, with higher transcription levels in PBMC (p-value=0.0005) of pre-treated cases as compared to healthy controls (Figure 4b). The transcripts gradually declined upon treatment without any significant differences with healthy controls therefore, infection-induced alterations in cytokines led to HPN mediated iron storage in monocytes.

IL-10, being immunoregulatory cytokine that regulates HPN homeostasis, was elevated in PBMCs in pre-treated as compared to post-treated cases (p-value<0.0001) and healthy controls; splenic data supported the PBMCs data for pre- and post-treated subjects (p<0.0001), with almost similar levels of transcripts in PBMCs and spleen (Figure 4c). The pathological consequences of the disease can be further supported by the inverse correlation between the IL-10 transcript and the hemoglobin levels (Figure 5a), which further added another dimension to establish the involvement of this anti-inflammatory cytokine in VL pathogenesis. Similarly, the regulatory effects of HPN were further identified in correlation studies where HPN expression was negatively correlated with the hemoglobin levels in active (pre-treated) cases (Pearson's $r=0.6598$, $p=0.004$) (Figure 5b). Since, iron also serves as the catalyst for iNOS production, direct correlation between the hemoglobin levels and the iNOS transcripts (Pearson's $r=0.5549$, $p=0.0257$) (Figure 5c), further consolidates the study regarding the role of iron in determining the immune responses against the intracellular parasite.

Discussion

Major source of iron in human is found as heme moieties in hemoglobin. Iron deficiency/excess, are associated with cellular and organ dysfunction. The causes underlying the ACD have been obscuring and not much has been known however, the increased uptake and retention of iron within cells reduces the functional iron required for production of hemoglobin. This in turn reduces the availability of oxygen to tissues leading to anemia.

We took the advantage of availability of spleen aspirates from VL patients, containing parasitized macrophages to directly address the possible function of iron since spleen is the major regulatory site for iron homeostasis. Direct analysis of transcriptional signatures of iron homeostasis and their role in context of the efficiency of cellular immune responses and disease pathogenesis was studied in pre- and post-treated splenic samples which was further extended in PBMCs of VL pre- and post-treated cases and endemic healthy controls; to specifically identify the cell subsets involved, the results were validated by single-cell specific transcriptional analysis of macrophages and T-cells for iron homeostatic transcripts.

Considering 93 VL cases admitted for the study with anemia and leucopenia as the key manifestations, we sought to establish relationship between leukocyte count and hemoglobin levels in VL cases who were not receiving any anti-leishmanial treatment at the time of

study. The significant positive correlation between leukocyte count and hemoglobin levels indicated for iron/hemoglobin levels as one of the crucial factors affecting leukocyte counts thus, establishing the link between the of severity of symptoms in terms of hemoglobin levels on leukocyte counts. Interestingly, no differences in the iron binding capacities(TIBC) indicated for the involvement of intricate network of molecular cascade that regulates immune homeostasis i.e. restoration of the normal immune functions. This could potentially serves to determine the immunological state of individual and development of cell-mediated immunity as reported previously by several authors ^{24,25}. Several other studies have established the association between iron status and immune response ^{26,27}, further establishing for the involvement of iron in regulating immune homeostasis i.e. antigenic encounter raises specific immune responses which aims at restoring the normal immune machinery. Also, ACD is accompanied with imbalance in the distribution of iron in body which causes ineffective utilization of iron for the formation of new blood cells.

To further delineate the affect of iron on immune homeostasis, relationship between the parasite specific antibody titres and hemoglobin was determined. The inverse correlation between two parameters indicated for the increased anti-leishmanial antibodies against the parasite decreased hemoglobin levels in circulation. Overall, presence of iron in hemoglobin plays important role in determining immune cell responses, thus, serves as determinant of disease severity.

Indeed, during intracellular infection, iron withholding is reinforced by the host, thus, genes involved in iron acquisition determines the disease pathogenesis and host immune responses ²³. Thus, the investigation of transcriptional profile of iron uptake and homeostatic mediators, revealed dynamic changes in some transcripts-as HPN and TfR1 to be associated with the spleen, reflecting the established line for spleen, being the primary site for iron homeostasis (iron uptake and recycling) usually from degenerated RBCs.

The gene transcripts associated with iron acquisition- DMT-1 and TfR1, were unaltered during the course of VL but TfR1 was significantly higher in spleen at pre-treatment than post-treatment which could be attributed to the essential role of this transporter in the spleen iron regulation as previously reported ²⁸. However, the relative expression in PBMCs was higher than in the spleen, expression trend in PBMC was further reflected in the monocyte/macrophage population, in accordance with the reported study from Nairz *et al.*²⁹. There are reports from *Legionella*, justifying for the decreased levels of TfR1 in activated macrophages ³⁰ which could be attributed to the presence of parasite and inflammatory environment mediated by IFN- γ ³¹. The altered transcript levels of TfR1 during VL, but no changes in transferrin-binding capacities (TIBC) between the controls and cases established the fact for reduced iron uptake during VL. This could be attributed as a consequence of transcriptional regulation of iron homeostasis, instead of any physiological alterations in the iron binding/carrying capacities. Indeed, this could be the consequence of different strategies the cellular machinery employs, to limit the availability of iron for parasite proliferation, in terms of TfR1 and DMT1.

Iron homeostasis in immune cells in terms of the transcripts of iron-regulatory hormone HPN and its effector molecule FPN, indicative of the parasite adaptive strategy against the

cellular machinery which causes iron redistribution within the PBMC and spleen. HPN levels were elevated in PBMCs as well as spleens, also, monocytes had elevated transcripts than the T-cells, reflecting the macrophage iron retention armory for exploitation by the parasite for its own benefit. Previous studies have validated the correlation of HPN with the blood-stage infection levels in *P. berghei* infection³², supporting our finding of inverse correlation of HPN with hemoglobin during VL, therefore, parasite re-engineers intracellular milieu to benefit its growth. HPN, small antimicrobial peptide and the regulator of innate immune response moved to center stage because of its role in regulating immune cell proliferation at least in lymphocytes. HPN-mediated alterations in the iron status can be said to result from downregulation of FPN in immune cells as reported earlier^{11,16}, increasing the availability of iron for parasite replication. This can be seen in the form of decreased FPN and increased HPN during VL. This can be further justified as the cause for the observed association between the spleen size and HPN therefore, HPN might play role in establishment of infection. Elevated HPN may also be involved in pathogenesis of disease in the form of VL-associated anemia³³. Interestingly, the decline in haemoglobin levels was directly associated with the observed increased levels of HPN during VL in our study. Also, HPN mediated iron loading onto macrophages/monocytes may result in iron mediated loss of effector functions therefore, providing a safe house for the intracellular pathogens as seen in Mycobacterium infection³⁴. HPN degrades FPN in macrophages and lymphocytes^{16,35} increasing availability of iron for parasite replication as in HIV. The regulatory effects of HPN were further revealed in correlation studies where its expression was negatively correlated with the hemoglobin levels in pre-treated cases, as reported from mice model^{36,37}. Although HPN regulatory pathways are complicated at molecular levels however, different physiological and molecular parameters report for HPN expression in lymphocytes of granulomatous pouch model of *E.coli*. have suggested for its involvement at site of infection³⁸ which was subsequently seen in macrophages and neutrophils³⁹ as evidence of key molecule determining the availability of iron to pathogen at foci of infection.

We found no differences in the transcript levels of FPN in PBMC and spleen, between different groups (D-0, Dis and EHC), which was different from study in *L. amazonensis* infected mice where FPN expression was inhibited³³. It could be attributed to the differences in tissue localization, cell-specific upregulation, chronicity of visceral form of disease along with additional factor of post-translational regulatory mechanisms imposed by HPN for FPN degradation⁴⁰. However, PBMCs had lower FPN transcripts as compared to the spleen, which can be attributed to the higher levels of HPN in the PBMC; therefore, retaining iron within the cell and its utilization by the parasite causes perturbation in cellular iron pool, bestowing in immune dysfunctions⁴¹ and disease exacerbation. HPN up-regulation during parasitic infection in human cells has to our knowledge never been formally reported, moreover, iron retention and immune inhibitory effects of iron has been known but little is known about its impact on immune functions.

Previous research has showed compromised iron homeostasis in M2 macrophages had resulted in dysregulation in proteins involved in antigen presentation, thereby reducing the effectiveness of the immune response⁴². This suggested for a potential immunoregulatory role of iron homeostasis by HPN to encourage macrophage activation for T-cell recognition and proliferation. This opened up another aspect to explore the HPN capacity and its

potential. Iron has been known to negatively regulate macrophage/monocyte activating potential of IFN- γ . This in turn hampers its effector functions -antigen presentation, reduction in TNF- α production and anti-microbial functions⁴³. Our initial findings led us to look upon for HPN-mediated iron retention and its immune inhibitory effects in VL, in terms of the immune activation marker HLA-DR, which was significantly reduced during VL. The assessment of immune activation marker HLA-DR, revealed for significantly reduced transcripts levels in VL cases as compared to controls without any significant differences in PBMC and spleen. It could be assumed as a consequence of iron-induced suppression of the immune stimulatory effects of IFN- γ on antigen presentation by HLA-DR. It goes in line with *in vitro* model of human cell line ⁴⁴, and *Salmonella* model ²⁹ where a cascade of negative regulatory events were responsible for compromised effector functions as RNS formation and TNF- α production ⁴⁵. Similarly, iNOS, heme-containing effector molecule of innate immune armory, expression was also compromised during infection, but no significant differences were observed in spleens of the VL cases (pre- and post-treatment), possibly due to residual infection in the spleen upon completion of treatment tenure; higher transcriptional levels in PBMCs of post-treated cases, signified the improvement in hemoglobin levels upon cure, as a carrier of iron that serves as catalyst for NO production by directly influencing the iNOS levels, as reported previously ³. However, no significant differences were observed in transcripts of iNOS in PBMCs and spleen, signifying the similar extent of compromised effector functions in spleen and PBMCs. Correlation study further validated the relationship between the hemoglobin levels and iNOS transcripts strengthening the established studies which have suggested for the role of iron in innate antimicrobial defense in tissue culture and animal model ^{3,46}. Further HPN-mediated immune modulatory effects are known to be directly orchestrated by pro- and anti-inflammatory cytokines as reported previously ^{12,47–49} therefore, we evaluated in terms of the expression of pro- and anti-inflammatory cytokines- IFN- γ , IL-6 and IL-10. IFN- γ transcript levels significantly upregulated during pre-treatment, PBMCs and spleen had almost similar levels of transcripts, these effects can be supported by the synergistic effects of IFN- γ on hepcidin expression in *Mycobacterium* infection ⁵⁰. The effect of iron on pro-inflammatory responses was further validated by transcriptional analysis of another inflammatory marker IL-6, which was also upregulated during active VL compared to post-treated and healthy controls, was indicative of infection induced inflammatory environment and was in line with studies from knockout model ⁴². Therefore, infection-induced alterations in cytokine profile led to HPN mediated iron storage in macrophages resulting in the pathological manifestation of anemia due to insufficient iron availability for erythropoiesis.

IL-10, being immunoregulatory cytokine that regulates HPN homeostasis, was elevated in splenic as well as in PBMCs of pre-treated subjects. The trend for IL-10 transcripts in VL was in line with malaria model, which could attribute towards the IL-10 dependent induction of HPN in peripheral blood derived monocytes/macrophages ⁴⁹ and in *Salmonella* infection system ⁵¹.

These findings imply that *L.donovani* infection may cause significantly reduced cellular iron uptake by downregulating TfR1 and DMT-1 in response to the immune stimulation by altered cytokine profile in terms of IFN- γ , IL-10 and IL-6. These set of events may

culminate in reduced iron availability to *L. donovani*, as an innate response mediated by immune cells, but the parasite exploits these immune stimulatory responses as regulatory factor for its own benefit, by employing HPN-dependent homeostatic pathways. This HPN-mediated pathway could abrogate the stimulatory effects of IFN- γ on antigen presentation by downregulating HLA-DR^{44,52} to perturb the cellular iron pool thereby, promoting IL-10 mediated anti-inflammatory responses during disease pathogenesis, as reported by Schaer *et al.*²¹. This effect may be assumed to attribute towards decreased innate effector responses, as documented by lowered levels of RNS by iNOS and affecting the macrophage polarization status⁴¹, thereby, subverting the immune machineries required for clearing off the intracellular invasion.

Finally, these findings in humans and other studies from human as well as mice model, warrant that HPN could serve in armory of surveillance as an important regulator of intracellular iron homeostasis. However, this field requires further attention to explore the extent to which hepcidin affects the immune regulatory pathways and the intricate network of iron regulation in clinical settings where HPN could serve as limiting factor, determining the innate and cell-mediated immune responses and therefore, proliferation and pathogenicity of parasite.

Exploitation of iron homeostatic pathway as a therapeutic option by depriving the parasite of iron and changing the cellular iron status in addition to the inhibition of HPN expression could serve as a complementary treatment option to limit parasite proliferation and enhancing the cellular cytotoxic mechanisms by enhancing the sensitivity of immune cells towards IFN- γ thus, improving their cytotoxic potential and cell-mediated effector mechanisms. As the current treatment options of VL has been obscuring due to one or the other factors which calls for development of newer strategies as targeting host iron metabolism to combat the issues related to disease burden, relapse rates and persistence of parasites even after cure. This could be of great therapeutic importance with the potential to affect disease pathogenesis.

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Abbreviations:

VL	Visceral Leishmaniasis
iNOS	inducible nitric oxide synthase
HPN	Hepcidin
TfR1	Transferrin receptor
DMT-1	Divalent metal transporter
FPN	Ferroportin

RNS	Reactive nitrogen species
KAMRC	Kala-Azar Medical Research Centre
PBMC	Peripheral blood mononuclear cells
TIBC	Total/transferring iron binding capacity
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-6	Interleukin-6
HLA-DR	Human leukocyte antigen
ACD	Anemia of chronic disease

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Figure 1.

Altered clinical profiles in *L. donovani* infection. (a) hemoglobin levels, (b) WBC count (per microlitre blood sample) in pre-(D-0) and post-treated (DIS) cases, (c) linear regression plot showing strong correlation between WBC counts and hemoglobin levels at pre-treated stage ($p=0.006$, Pearson's coefficient, $r=0.2991$) (d) serum/plasma transferrin iron binding capacity(TIBC) from pre-treated cases versus healthy controls, (e) linear regression plot showing strong inverse correlation between parasite-specific antibody titres with hemoglobin and (f) linear regression plot showing strong inverse correlation between spleen size(in centimeters) and hemoglobin levels. Comparison between pre (D-0) and post-treatment (D-Dis) were done using paired t-test. Comparison between VL and EHC was done with Mann-Whitney test. (ns=non-significant, ** $p<0.01$, *** $p<0.001$).

Figure 2.

Relative mRNA expression of iron homeostatic transporters. mRNA levels of iron transporters in PBMCs as well as spleen and their comparison; and purified immune cells (CD14+ and CD3+) (a) Transferrin receptor1(TfR1) transcripts of endemic healthy controls(EC), pre- treated (D-0) and post-treated cases(DIS) (after 30 days of treatment), (b) divalent metal transporter-1(DMT-1), (c) hepcidin (HPN) and (d) ferroportin(FPN). Each symbol represents one sample. Comparison between VL and EC were done using t-test and between cell populations originating from the same donors using a paired t-test. Non-parametric tests (Mann–Whitney U test or Wilcoxon matched pair test) were used when normality test failed. (ns=non-significant, ** $p < 0.01$, *** $p < 0.001$).

Figure 3.

Relative mRNA expression of major effector molecules in leukocytes of VL cases. mRNA expression of (a) HLA-DR in VL (pre- and post-treatment) and EC PBMC as well as splenocytes, and (b) comparative expression levels of iNOS in PBMCs and splenocytes. Comparison between VL and EC were done using t-test and between cell populations originating from the same donors using a paired t-test. Non-parametric tests (Mann–Whitney U test or Wilcoxon matched pair test) were used when normality test failed. (ns=non-significant, ** $p < 0.01$, *** $p < 0.001$). (c) linear regression plot for inverse correlation between hepcidin expression and hemoglobin levels ns= non-significant and significant differences are indicated by p-values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Figure 4.

Relative expression of effector cytokines in VL cases. (a) Relative mRNA expression of IFN- γ in PBMCs of pre- (D-0) and post-treated (DIS) cases as compared to healthy controls (EC) and splenocytes (b) mRNA expression of IL-6 in PBMCs and splenocytes and (c) mRNA expression of IL-10 in PBMCs and splenocytes. Comparison between VL and EC were done using t-test and between cell populations originating from the same donors using a paired t-test. Non-parametric tests (Mann-Whitney U test or Wilcoxon matched pair test) were used when normality test failed. Significant differences are indicated by P-values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Figure 5.

Linear regression analysis for correlation of hemoglobin levels with immunological parameters. (a) Inverse correlation observed between hemoglobin levels with IL-10 in PBMCs of pre-(D-0) ($p = 0.0105$) (b) Similar inverse correlation was observed with the mRNA levels of hepcidin ($p = 0.0040$) and (c) Direct correlations were observed between the hemoglobin levels with the transcript levels of iNOS ($p = 0.0257$). Pearson's analysis was performed for the correlational study. Significant differences are indicated by P-values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Table 1.

Clinical data for VL cases for the study

Clinical parameters	Mean±SD (Median)
Age	26.89±15.52(21.20)
Sex % (M/F)	60/40
Fever history (in days)	45.02±50.44(47.7)
Spleen size (in cm)	4.24±3.33(3.79)
Hemoglobin (gm/dL)	7.77±1.35(4.56)
Total/Transferrin-iron binding capacity (TIBC) (µg/dL)	402.94±41.20(416)
Serum iron (µg/dL)	127.65±34.96 (146)