ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

An approach to hemoglobin recycling through Leishmania infected macrophage and its impact on visceral leishmaniasis

Ajit K Thakur, Pushpanjali, Shyam Narayan*, Sanjiva Bimal, Subhankar K Singh, Anil K Gupta, Pradeep Das
Rajendra Memorial Research Institute of Medical Sciences, (Indian Council of Medical Research) Agam Kuan, Patna – 800 007, India.

Manuscript Info

Manuscript History:

Received: 12 December 2014
Final Accepted: 26 January 2015
Published Online: February 2015

Key words:

****Corresponding Author***
Shyam Narayan

Abstract

Leishmania, due to a biosynthetic deficiency characteristically requires exogenous heme for in vitro growth. Through present study, we have investigated the relevance of CD163 and HCP-1 in trafficking of Hb macrophages sensitized with L. donovani.. Over expression of CD163 on macrophage after its stimulation with parasite is crucial to enhance the adhesive forces to optimize Hb binding on parasite to ensure its survival. It was shown that stimulation with Leishmania donovani, macrophage in the presence of hemoglobin, could trigger efficient IL-10 production. This observation is relevant to the pathogenesis which is linked to direct effect of IL-10 on CD163 expression, which make availability of Hb/heme to the intracellular L. donovani which could contribute to the anemic condition in Kala azar infected patients.

INTRODUCTION

Leishmania donovani, a protozoan parasite is responsible for substantial public health problem affecting millions of people worldwide. The parasite is responsible for disease visceral leishmaniasis(VL) or Kala azar which is usually fatal if left untreated, 0.5 million cases of visceral leishmaniais are estimated every year of which 0.1 million are estimated in India. (Chang, 1983)

Chemotherapy is the only effective way of control against the disease. However, considering the huge toxicity of drug used in control of the disease, attention has recently been focused to find out new drug targets for control. As known *Leishmania* is devoid of heme biosynthetic pathway (Chakravarti et al., 1994; Sah et al., 2002) and such identification of factors promoting the heme dependency of parasite, can help in designing of new drug target. The majority of the heme is sequestered in erythrocytes in the form of Hemoglobin (Hb) and during hemolysis by several factors; the Hb which moves freely in the blood circulation is known to finally get attached to the macrophage through support provided by CD163 receptor on macrophage (Schaer et al., 2006). CD163 receptor, a single transmembrane domain expressed exclusively on human Monocytes and macrophages is a member of the cysteine rich scavenger receptor (SRCR) superfamily (Hintz et al, 2002). The relevance of CD163 has also been shown through infiltration of monocytes during the resolution phase of inflammatory reaction (Sulahian et al., 2000). In addition to CD163, Heme Carrier Protein-I (HCP-I) and the ferrous iron transporter DMT-I contribute to a spatially linked pathway for Hb/heme iron recycling in macrophages (Schaer et al., 2008).

Role of IL-10 in inflammatory responses is widely discussed in VL because of its capacity to down-regulate class II MHC expression as well as on inhibition of pro inflammatory cytokines by monocyte (Spits and De Waal, 1992). The Interleukin-10 was also reported to enhance the expression of CD163 receptor during the maturation of monocyte into macrophages *in vitro* (Sulahian et al., 2000). It was also suggested that Hp–Hb (heptoglobin-hemoglobin) binding to CD163 on human monocyte/ macrophage isolated *in vitro* and *in vivo* elicits a direct anti-inflammatory effect *via* the secretion of IL-10 (Philippisdis et al., 2004). For this reason it is anticipated that Hp–Hb binding *via* CD163 might enable

macrophages to coordinate for hemoglobin scavenging and its subsequent breakdown with anti-inflammatory activity. All these reports related to regulation of CD163 expression by pro- and anti-inflammatory mediators as well as secretion of anti-inflammatory cytokines in response to ligand binding to CD163 reflects towards a function of CD163 in immunomodulation and anti-inflammation (Lee and Chau, 2002).

In the present study we have investigated the relevance of CD163 and HCP-1 in trafficking of Hb macrophages sensitized with *L. donovani*. To highlight the importance of CD163 during Hb binding in VL, a neutralizing CD163 mAb was used as inhibitor and its effect on binding to Hb by *L. donovani* sensitized macrophage was investigated. We have also investigated the HCP-1 level in *L. donovani* sensitized macrophage which is involved in the macrophage Hb-iron recycling pathway. We demonstrated that Hb binding to CD163 influences the IL-10 production which is further enhanced in the presence of *L. donovani*. Further study is needed to see the pathway of CD163 endocytosed Hb up to the intracellular *L. donovani* via phagosome and phagolysosome in the case of VL.

2. Materials and Methods

2.1. L. donovani parasite

Leishmania donovani strain AG 83 (MHOM/IN/83/AG83), originally obtained from Indian VL cases was maintained in golden hamster (Sakcs and Melby, 1998). Triturated spleen from infected hamster was used to set promastigotes culture at 24˚C, RPMI-1640 (Sigma Aldrich, St.Louis) supplemented with 10% FCS, pH-7.4. 100 U penicillin, 50 µg streptomycin/ml and 50µg gentamycin. The cultured parasites were cryopreserved and revived before experimentation.

2.2. Samples from healthy control

To examine the alteration in terms of Hb binding response of macrophages, fourteen subjects of both sexes of all age group were enrolled in the study after taking their written informed consent.

2.3 Macrophage culture

Peripheral-blood mononuclear cells (PBMCs) were purified using Ficoll-Hypaque^{TM} (GE, Germany) density gradient centrifugation at 800xg, 15 min at 20 ˚C. Cells were suspended in RPMI-1640 medium (Sigma Aldrich, St.Louis) with 10% FCS (Sigma Aldrich, St.Louis), dispensed into tissue-culture grade-Petri dishes (Nunc, Denmark) and incubated at 37^0C in a humidified, 5% $C0_2$, 95% air, for 3h. Non-adherent cells were then removed by washing, twice with, pre-warmed HBSS. Fresh pre-warmed RPMI-1640 with 10% FCS was added to each dish before the adherent cells were sensitized with *L. donovani* promastigotes and incubated at 37^0C in CO_2 incubator. The 72h adhered and stimulated cultured cells were then gently scrapped off and re-suspended in fresh medium. As, morphologically almost all (92%) of the cells left in the dishes appeared to be macrophages. Cells were gently scrapped off and transferred to a 5 ml tube. Monoclonal anti-human-CD14 antibody (BD-USA) was added to each sample of macrophage at 5 µl (5µg) per tube to identify the purity of monocytes purified by PBMCs by flow cytometry.

2.4 Hb estimation in macrophage

Macrophages were stimulated in 12 well tissue culture plates with $2x10^6$ *L. donovani* promastigotes in ratio 1:20 for 2h at 37˚C. Control culture was set up in medium alone. Estimation of binding of Hb to macrophage was made as previously described (Sengupta et al, 1999). Briefly, macrophages ($2x10^6$/ml) in each dish were incubated with 50µg/ml Hb sol^n (4˚C, 30 min). After washing, harvested cells were consequently co-incubated with human anti Hb-FITC(Santa cruz, USA) in PBS for 30 min at 37˚C which allowed for detection of Hb binding. Cells were then washed and re suspended in 500µl stain buffer containing 1% fresh prepared formaldehyde (Merck, BDH, India) for estimation of fluorescence in FACS caliber. Flow data on binding of Hb on macrophage were evaluated on cell pro quest flow software. Negative control sample were incubated with relevant iso match antibodies FITC (FITC and PE incubated IgA pharmingen, USA) in parallel with all experimental sample.

In subsequent experimentation, attempt was made to abruptly disrupt Hb trafficking by treating the sensitized and non-sensitized macrophages with CD163 receptor blockade to find out reversal if any, in binding efficiency in the

macrophages. Briefly, *L. donovani* sensitized or unsensitized macrophages ($2x10^6$/ml) were either left untreated or treated with CD163 receptor blockade (4˚ C, 30 min) prior to their co-incubation with 50 µg/ml Hb sol^n (4˚ C, 30 min). The binding of Hb was re assessed as described above.

2.5. IL-10 Cytokine detection

One million ($1x10^6$) PBMCs were cultured alone or with *L. donovani* at a 1:1 ratio with or without Hb at 50 µg/ml for 18 h. Supernatants of these co-cultures were harvested, and Interleukin-10 (IL-10) concentration was determined in separate experiments by the IL-10 ELISA test kit(BD Opt EIA, USA). The assay was performed as described in the instructions. Absorbance at 450 nm was determined on a microplate reader (Bio-Rad). Sensitivity limit of the ELISA for IL-10 was 4.0 pg/ml. Samples were assayed in triplicate. Results were analysed by Repeated measure ANOVA

2.7 Expression of CD163 in monocyte

Peripheral-blood mononuclear cells (PBMCs) were isolated according to standard protocol mentioned above. Macrophages were stimulated in 12 well tissue culture plates with IL-10, IL-10+LD, LD alone. The cells were then stained for 15 min on ice with saturating concentrations of the following fluorochrome-conjugated antibodies: anti-CD14 as a fluorescein isothiocyanate (FITC) conjugate, anti-CD163 as an phycoerythrin (PE) conjugate from PharMingen (Germany), conjugate from Becton Dickinson (Germany). After subsequent washing steps the fluorescence signals were detected by a FACS *Calibur* flow cytometer (Becton Dickinson). The data were analyzed and expressed as mean fluorescence intensity by using the Proquest software (Becton Dickinson). The cells were not permeabilized and only the surface expression of CD163 was analyzed.

2.6. RNA isolation and quantitative real-time RT-PCR of CD163 & HCP-1

Total cellular RNA from human blood-derived macrophages was isolated using the TRIZOL method. Equal amounts of total RNA was used to prepare cDNA using Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen,USA) according to the manufacturer's instructions**.** The cDNA was quantified spectrophotometrically using Nanodrop (Thermo Fisher). Duplicates of cDNA samples were amplified by Real Time-PCR using the ABI 7500™ real-time PCR system with Quanti Tect SYBR Green- I kit (Qaigen, Germany) and sequence-specific primer pairs for CD 163 (Forward 5' ATG TGA TGA CGG CTG GGA CAG TTA 3', Reverse 5 'GCA AGA AAC GCT GTC AAG CCA GAT 3'), HCP-1 (forward 5'AAC TCA CTC TAC CCA GCC ACT C-3, reverse 5'ATC AGC CTT TTC CAG CAT CCC-3')and GAPDH (forward 5-AAC AGC GAC ACC CACTCC TC-3, reverse 5 -GGA GGG GAG ATT CAG TGT GGT-3) Primers were designed using Primer Selection software (Primer Quest). Melting curve analysis was performed simultaneously in each PCR experiment to detect primer-dimer formation and the specificity of each amplicon. Temperature cycling profiles were as follows: initial denaturation of 15 min at 95°C, followed by 40 cycles of denaturation of 30 s at 95°C, annealing of 15s at 63-55°C, and extension of 15s at 72°C. For CD163 primers, Real-time PCR results were analyzed with 7500 V.2.0.3 (ABI 7500, USA). Final data were expressed as mRNA expression in treated cells relative to the expression level in untreated cells. Expression levels of CD163 and HCP-1were normalized to GAPDH levels in each experimental sample. Changes in mRNA expression were analyzed for significance by one tailed student t-test.

3. Statistical analysis

Results are expressed as mean plus or minus SD and are derived from multiple independent experiments, as indicated in the figure legends. Data were analyzed by one-way analysis of variance (ANOVA), Repeated Measure ANOVA, Paired *t*-test (one tailed) for differences of mean of three independent samples and appropriate post tests were carried out using Graph Pad Prism 5.0 software.

4. Results

4.1 Qualitative evidence for enhanced binding of Hb by macrophage

Data presented in Fig-1 revealed an increased tendency of the macrophage to show an affinity binding of Hb up to 26.3%. There was a clear indication that *Leishmania* dictated increase in CD163 expression and Hb binding since attachment of Hb was only up to 16.1% in macrophages in the absence of *L. donovani* sensitization. This illustrates an increased tendency of Hb attachment to the *L. donovani* sensitised macrophage via CD163 which might be due to

enhanced expression of CD163 in the influence of *L. donovani*. Subsequent result showed that this ability of *Leishmania* to drive greater proportion of macrophage to bind with Hb was effectively inhibited in the presence of CD163 receptor blockade (17.9 %). Fig 1(inset) is a representing flow cytometry data illustrating an increased Hb binding efficiency of macrophage in presence of *Leishmania* as well as a reversal in this trend after co-incubation of macrophage with anti CD163 receptor blockade. Student-Newman-Keul test was performed using Graph pad prism software.

4.2 Increased utilization of Hb by macrophage

To further examine the possibility for an increased binding of Hb by the *L. donovani* infected macrophage parasite, we determined the reduction in the quantity of Hb after a coincubation of macrophage with parasite by Hb assay method. Briefly, Macrophage culture were either treated with *L. donovani* or were left untreated. In parallel experiment was set for a co-incubation of macrophage with anti CD163 to asses the extent at that inhibition could act upon to bring a reduction in the ability to bind to Hb. The supernatent from the culture were examined for Hb level through spectrophotometric method. As shown in Fig-2, level of Hb which was observed in macrophage culture supernatant to be 35.4µg/ml indicated that a considerable amount of Hb was utilised by macrophage in comparision to control, which comprised Hb only. In contrary there was observed a reduction in Hb level after a co-culture of macrophage with *L.donovani* in the culture supernatant. The Hb level become as low as 27µg/ml in such culture condition, which was a reflection for an increased use of Hb by the *Leishmania* parasite. This reduction in Hb level was observed to be significantly low in *L. donovani* treated macrophage compared to Hb control ($P<0.002$) as well as compared to macrophage which were not sensitized to *L.donovani* . This trend reverted after an addition of anti CD163 receptor in the *L.donovani* sensitized macrophase and as such, there was an increase in the yield of Hb, upto 48.2µg/ml in culture supernatant.

4.3 Leishmania infection is linked to over expression of CD163

In order to determine whether the increased expression of CD163 on monocytes is due to increased RNA and protein synthesis, we analysed mRNA expression of CD163. Semi quantitative PCR of mRNA isolated from macrophages either *L. donovani* sensitized or non-sensitized indicated that there was an increase in mRNA expression level of CD163 (Fig-3). We further carried out investigation on relative quantity of specific mRNA in *L. donovani* sensitized cells in relation to untreated cells by Real Time PCR. For the experiment mRNA were normalized to GAPDH. The Real Time PCR results obtained in presence of 180 bp GAPDH later corroborated the significantly over expressed CD163 in macrophage after *L. donovani* stimulation ($P=0.001$). Addition of *L. donovani* in the macrophage culture induced the expression of CD163 receptor on the surface of these macrophages significant compared to untreated macrophage cells. (Fig-4)

4.4 Under expressed HCP-1 helpes in an increased Hb inside during VL

We studied the regulation of HCP-1 by *L. donovani*. After 12 hr, we observed 1.5 fold down-regulation of HCP-1 mRNA in the presence of *L. donovani* (Fig-5). Down regulation of HCP-1 restricted the transport of Hb-derived heme out of the endosomes into the cytoplasm. One might speculate that suppression of HCP-1 could impair the transport of heme from extracellular sites or from Hb-heme, which was endocytosed by CD163, to the microsomal heme oxygenase; this could facilitate the endocytosed Hb inside the endo-phagosome for the intracellular *L. donovani*

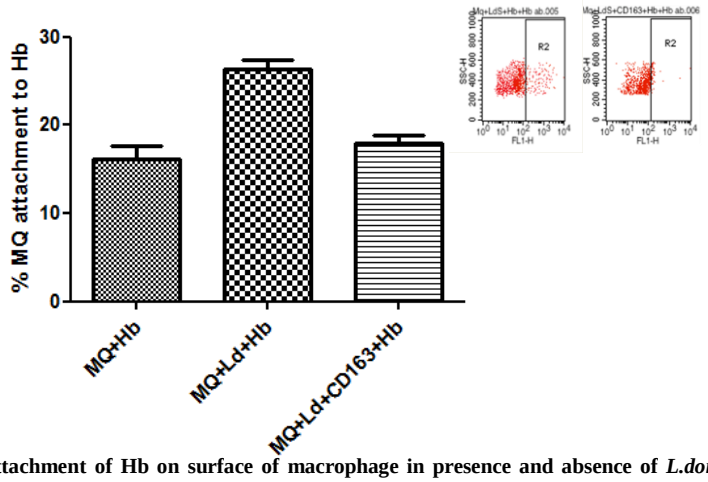
4.5 IL-10 analysis

Because of the fact that, the increased utilization of Hb by the *Leishmania* in the macrophage, promoted clinical situation such as VL; we questioned, whether or not such a unique tendency of *Leishmania*, adversely influence immune response in the VL patients. We hence investigated IL-10 cytokine pattern, because previous study from India document the key role of IL-10 in the pathogenesis of this disease (Thakur et al, 2003). For this $1x10^6$ PBMCs were cultured alone or with *L. donovani* at 1:1 ratio with or without Hb at 50 µg/ml for 18 h. As shown in Fig-6 the level of IL-10 suddenly increased after *in vitro* stimulation with *L. donovani* in the presence of Hb compared to control ($P<0.001$). This observation was relevant to the pathogenesis. Since, stimulation of *L. donovani* led to an increase in IL-10 (59 pg/ml) but the up regulation in IL-10 production was clearly higher by the *Leishmania* parasite at the time when Hb actively utilized by the parasite. Pairwise comparisons were done using repeated measure ANOVA (Newman-Keul Multiple Comparison Test).

4.6 CD163 expression in the presence of IL-10

In order to determine the level of CD163 expressed by macrophages, PBMCs were cultured for 7 days in either the presence IL-10, IL-10+LD and LD alone respectively. Macrophages were then analysed for CD163 expression by flow

cytometry. As seen in Fig-7 there was a dramatic increase of up to 2 fold in CD163 expression on macrophages in the presence of IL-10 and LD.



Attachment of Hb on surface of macrophage in presence and absence of *L.donovani* before and after blocking of CD163 Receptor

Figure-1-- legand shows increased binding of Hb to macrophage cells following sensitization with *L.donovani*. Macrophages (2x 10^6/ml), isolated from peripheral mononuclear cells were challenged with promastigotes and were co-incubated with 50 µg/ml Hb sol^n (4˚ C, 30 min).The binding of Hb was assessed on the basis of fluorescence intensity of cells using flow cytometry. One way ANOVA for differences of mean of three independent samples was used to compare data between the two groups. Inset: A representative flow diagram of untreated treated and blocked receptor showing difference hemoglobin attachment.($P<0.01$)

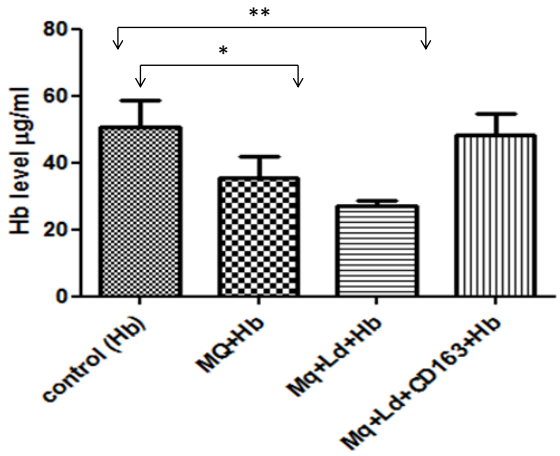


Figure-2. Comparative bar diagramme showing hemoglobin concentration in culture supernatant PBMCs treated with or without *L.donovani* and anti CD163 antibodies. Concentration of Hb found to be only 27µg/ml in *L.donovani* sensitized macrophage culture supernatant in comparison to control Hb which was 50 µg/ml ($P<0.002$). Hb utilization is restricted in the presence of CD163 receptor blocked antibody.

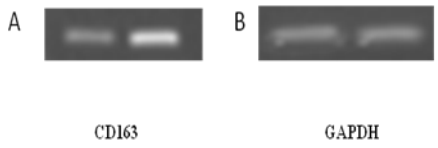


Figure-3. (A) Semi quantitative PCR for CD163 m RNA expression in macrophage. lane 1- macrophage infected with *Leishmania donovani*. lane 2- *Products were separated on* agarose gel *and* stained with Ethidium bromide. (B) The 180 bp fragment of GAPDH.

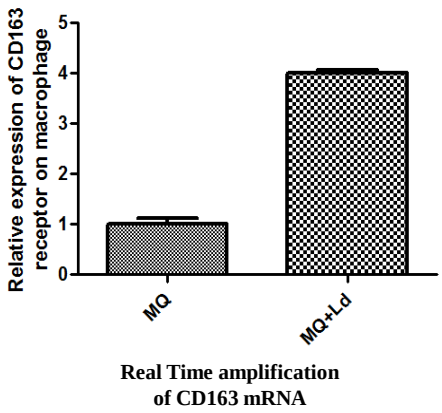


Figure-4. mRNA expression level of CD163 receptor on macrophage after treatment with *L.donovani* were measured by real time PCR. Results were presented as the relative quantity of specific mRNA in relation to untreated cells. mRNA were normalized to GAPDH. Means ±SD of at least three independent experiments per treatment and time-point were given significant changes of gene expression ($P > 0.001$).

Real Time amplification of HCP-1 mRNA

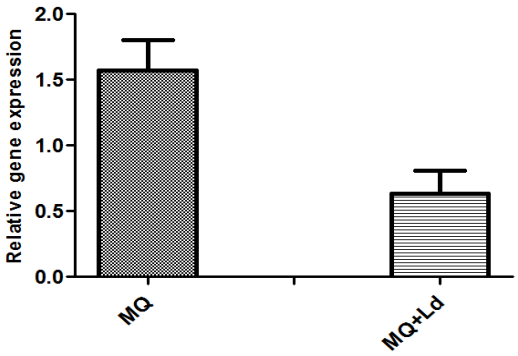


Figure-5 mRNA expression level of HCP-1 receptor on macrophage after treatment with *L.donovani* were measured by real time PCR. Results were presented as the relative quantity of specific mRNA.

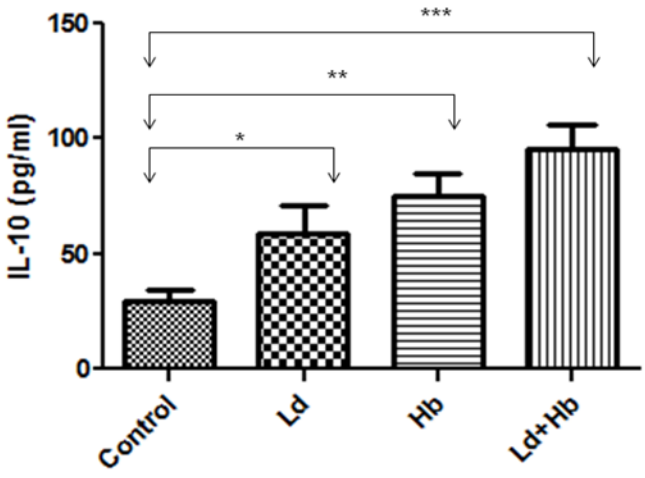


Figure-6 Supernatants from Macrophages cultured for 18h were analyzed for IL-10 by ELISA Bar diagram shows mean concentrations of IL-10 in triplicate culture supernatants ± SD, for a representative of n = 5 replicate experiments. Repeated Measure ANOVA was done using Graph Pad Prism Software.

˚ P< 0.05, ** P< 0.01, ***P<0.001

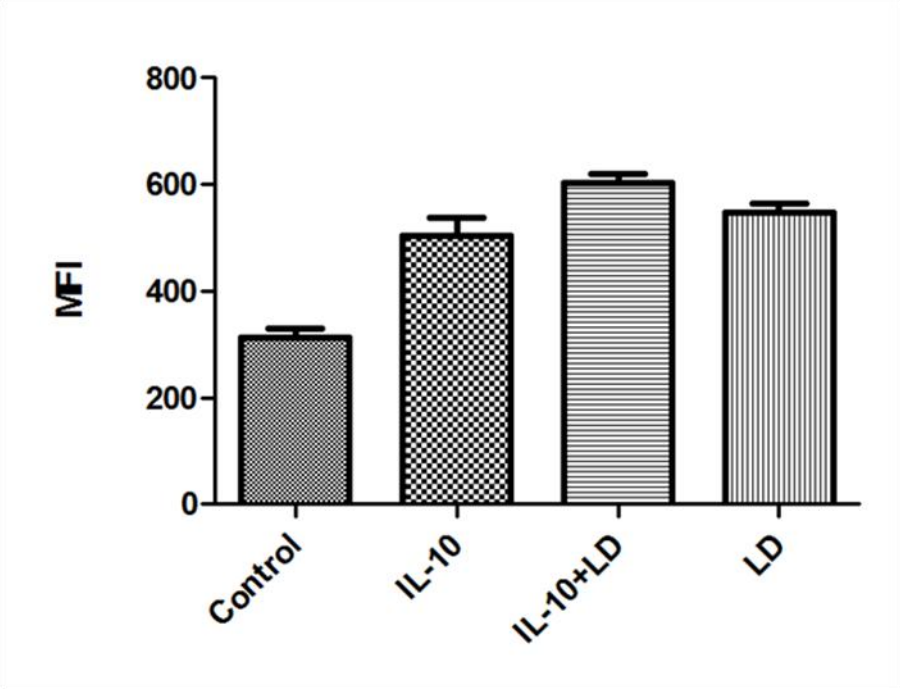


Figure-7- Showing Mean flourecence intensity for CD163 expression on Macrophage in response to IL-10 and LD

5. Discussion

CD163 is of special interest because its expression is restricted to monocytes/macrophages which were the prime host for *L. donovani.* The present study furnished strong indication that *L. donovani* affectes the CD163, HCP-1 spatially linked pathway for Hb/heme iron recycling pathway in macrophage. This was shown through pronounced tendency of *Leishmania* to over express the CD163 receptor. This increased expression of CD163 and Hb binding by *L. donovani* sensitized macrophage might be an additional mechanism by which *L. donovani* utilizes host Hb which might contribute in severe form of anemia and VL pathogenesis. In support of this assumption, previously it was shown that *Leishmania* endocytosed hemoglobin (Hb) through a high affinity receptor (HbR) located on cell surface (Sengupta et al., 1999) and that internalized Hb is targeted to the lysosomal compartment by Rab-5 and Rab-7 dependent endocytosis pathway (Patel et al.,2008).

Interestingly, the binding of Hb on macrophage in the presence and absence of *Leishmania* was different due to an over expressed CD163 receptor. It appears that an increase in expression of CD163 might enhance the binding and availability of Hb towards parasite which is crucial for its survival and pathogenesis. Given that Macrophages constitute the major cellular compartment for hemoglobin (Hb) degradation and subsequent recycling of heme-iron to erythropoesis and Heme Carrier Protein (HCP-1) spatially interact with CD163 to comprise the only known pathway for uptake of cell-free Hb as well as for Hb bound to the plasma protein haptoglobin. Over expression of CD163 on macrophage surface in the presence of *L. donovani* could impairs this recycling pathway and this deregulation of macrophage Hb/iron recycling pathway may contribute to major pathogenic mechanism of anemia associated with chronic disease (Schaer et al, 2008) and also correlates with degree of anemia (Thakur et al., 2013).

Other studies suggest that anemia is frequently observed in patients suffering from chronic inflammatory disorder (Tilg et al, 2002). IL-10 homeostasis lead to reduced serum iron concentration and transferrin saturation, where as ferritin level, reflecting body iron stores are up regulated (Means and Kranz, 1992). Additionally, it was shown that, a diversion of iron traffic might lead to withdrawal of iron from the site of erythropoesis and the circulation to the storage compartment in the macrophage. Several cytokine have been reported to affect iron homeostasis, in one study a dose response relation

was shown between IL-10 and CD163 expression (Satoskar et al, 2000). As such to validate the significance of CD163 in the increased uptake of Hb by *Leishmania*, it was important to establish that after stimulation with *L. donovani*, the parasite could trigger efficient IL-10 production. After stimulation with *L. donovani* in macrophage in the presence of Hb, cells behaved differently and produced significantly higher amount of IL-10. In contrast, macrophage from control group, which were not treated with *L. donovani* generated less IL-10. As the control group was not exposed to the parasites, there was no question of any immune impairment and as such macrophages produced less IL-10.

In summary our data provided a strong indication that CD163 is over expressed at the surface of macrophage after stimulation with *L. donovani* and in the presence of Il-10 which enhances CD163 mediated binding of Hb. We have also shown that expression HCP-1 is down regulated in macrophages in the presence of *L. donovani.* This endocytosed Hb by macrophage may be utilized by intracellular *L. donovani* which could contribute to the anemic condition in Kala azar infected patients. This can be mainly link to direct effect of IL-10 and *L. donovani* on CD163 and HCP-1 expression with activated macrophage which may facilitate the availability of Hb/heme to the intracellular amastigotes within macrophage.. Further studies are required to address how enhanced intake of Hb is reaching up to the intracellular *Leishmania* amastigotes and endocytosed by it, which can be exploited for the control based study in Visceral Leishmaniasis.

Acknowledgements:

Authors are thankful to Mr. S.K. Chaturvedi and Mr.B.N.Roy for their technical assistance.
Financial Support
RMRIMS (ICMR)
Competing Interests: None declared
Ethical approval: Ethical approval was obtained from the Institute Ethical Committee

References

1. Chakravarty, R., Sharma, M,C., Gupta, A,K., Prakash, N. and Saran, R. *Leishmania donovani*, Homolytic Activity of promastigotes, Experimantal parasitol. 1994; 8: 253-58.

2. Chang, K.P. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int Rev Cytol Suppl. 1983; 267-305.

3. Hintz, K.A., Rassiar, A.J. and Wardel, K. Endotoxin induces rapid metallo protease mediated shedding followed by up regulation of the monocytes haemoglobin scavenger receptor CD163. J Leukoc Biol 2002; 72: 711-17.

4. Lee, T.S. and Chau, L.Y. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med. 2002;8: 240-46.

5. Means, R.T. and Kranz, S.B. Progress in understanding the pathogenesis of the anemia of chronic diseases. Blood. 1992; 80:1639-47.

6. Patel, N., Singh, S.B., Basu, S.K. and Mukhopadhyay, A., Leishmania requires Rab7-mediated degradation of endocytosed hemoglobin for their growth. Procedings National. Acadamy of Science.U.S.A. 2008; 105, 3980–3985.

7. Philippidis, et al. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: anti-inflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ. Res. 2004; 94**:**119–26.

8. Sacks, D. and Melby, P. Animals models for the analysis of immune responses to leishmaniasis. In: Coico R, editor. Current protocols in immunology. Vol 4. New York: John Wiley & Sons; 1998.

9. Sah, J.F., Ito, H., Kolli, B.K., Peterson, D.A., Sassa, S. and Chang, K.P. Genetic Rescue of *Leishmania* Deficiency in Porphyrin Biosynthesis Creates Mutants Suitable for Analysis of Cellular Events in Uroporphyria and for Photodynamic Therapy. J Biol Chem. 2002; 17: 902-09.

10. Satoskar, A.R., Rodig, S., Telford, S.R., Satoskar, A.A and Ghosh, S.K. IL-12 gene-deficient C57BL/6 mice are susceptible to *Leishmania donovani* but have diminished hepatic immunopathology. Eur J Immunol. 2000; 30: 834–39.

11. Schaer, C.A., Schoedon, G., Imhof, A., Kurrer, M.O. and Schaer, D.J. Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. Circ. Res. 2006; 99: 943–50.

12. Schaer, C.A., Vallelian, F., Imhof, A., Scheodon, G. and Schaer, D.J Heme carrier protein (HCP-1) spatially interacts with the CD163 hemoglobin uptake pathway and is a target of inflammatory macrophage activation. J Leukoc Biol. 2008; 83: 325-33.

13. Sengupta, S., Tripathi, J., Tandon, R., Raje, M., Roy, Basu, S.K. and Mukhopadhyay, A. Hemoglobin Endocytosis in *Leishmania* is Mediated Through 46 kDa protein Located in the Flagellar Pocket. J Biochem Chem. 1999; 274: 2758-65.

14. Spits, H. and De, Waal. Malefyt R Functional characterization of human IL-10. Int Arch Allergy Appl Immunol. 1992; 99**:**8–15.

15. Sulahian, T.H., Hogger, P., Wahner, A.E., Wardwell, K., Goulding, N.J., Sorg, C., Droste, A., Stehling, M., Wallace, P.K., Morganelli, P.M., Guyre, P.M. Human Monocytes Express CD163 Which is up regulated by IL-10 and identical to p155. Cytokine. 2000; 12: 1312–21.

16. Thakur CP, Mitra DK, Narayan S. Skewing of cytokine profile towards T helper cell type 2 response in visceral leishmaniasis patients unresponsive to sodium antimony gluconate. Trans R Sac Trop Med Hyg. 2003; 97: 409-12.

17. Thakur, A.K., Bimal, S., Singh, S.K., Gupta, A.K., Das, V.N.R., Das, P., Narayan, S. Degree of anemia correlates with increased utilization of heme by *Leishmania donovani* parasites in visceral leishmaniasis. Experimental Parasitol. 2013; 135: 595–598.

18. Tilg et al. Treatment of Crohn's disease with recombinant human interleukin 10 induces the pro-inflammatory cytokine interferon y. Gut. 2002; 50:191-95.