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INVESTIGATION OF sHLA-G AND IT'S RECEPTOR (LILRB4) IN IRAQI PATIENTS INFECTED WITH *L. INFANTUM* AND THEIR EFFECTS ON THE LEVEL OF IL-12

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Abstract

Visceral leishmaniasis(VL) or kala-azar is one of the world most neglected tropical diseases in mortality and fourth in morbidity, rK39 dipstick was used to diagnose the suspected infected patients as easiest and rapid technique for VL diagnostic, the disease out-coming required to the differentiation of cell mediated immunity either T-helper 1(Th-1) or (Th-2). One of main pointers that may be considered as one of immune evasion strategy in the host-parasite interplay is HLA-G level alteration. HLA-G Known as a special proteins (non-classical HLA class I) molecules which can suppress the immune system by T-cell functions impaired in the aid with target receptors as LILRB4. The development of the cell mediated immunity initiated with Interleukin-12(IL-12) production by antigen presenting cells (APCs) that induce Interferon-^{3} (IFN-^{3})-secreting (Th-1) T cells. The sudden modification in IL-12 level may be referred to some-thing wrong occurred in *vivo* and the induction may be positively or negatively to naïve T-lymphocytic cell activation as immunity response. So, this study aimed to investigate the alteration in sHLA-G and its receptor levels could be impressed the mean level of IL-12 in VL patients, also tried to finding any correlation between them. All patients scored high significant ($P< 0.01$) increase level in sHLA-G and its receptor with mean of (17.951± 7.78 ng/ml) for sHLA-G in comparison to control group (0.177± 0.12ng/ml) and high significant ($P< 0.01$) expression of LILRB4 receptor for sHLA-G on different immune cells (5.149 ± 2.043ng/ml) in comparison to control group (0.279± 0.012 ng/ml). While, the result showed high significant increased ($P<0.01$) of IL-12 (249.094± 79.37 pg/ml) in comparison to control group (25.079 ± 3.19 pg/ml) with significant linear positive correlation between this cytokine and sHLA-G and its receptor.

Key words : Visceral leishmaniasis. VL diagnosis, MHCG1, sHLA-G, LILRB4, IL-12, Elisa assay.

Introduction

Visceral leishmaniasis (VL), classified as a Neglected Tropical Climate Disease (NTD) (WHO, 2017). Characterized by acute, sub-acute or chronic, disease (Pelissari *et al.,* 2011).

VL caused by the infective stage (promastigote) of obligate intracellular parasite belonging to genus *Leishmania*, as *Leishmania donovvani* in human and *Leishmania infantum(chagasi)* in both human and dogs (Bankoti and Stager, 2012).

Bone marrow, spleen and liver are the main organs for *Leishmania*'s establishment. The incubation period ranged from (2 week-18 months), associated with scandalous inflammatory in viscera swelling through (2-8) months after infection (Ready, 2014).

The clinical signs includehepato-splenomegaly, long-term and low-grade fever, anemia, leucopenia, hyper-gamma-globulinemia, cachexia, weight loss, (Karunaweera and Ferreira, 2018).

The up-regulation in HLA-G expressed clearly in a various pathological condition such as visceral leishmaniasis, cancers, viral infection, organ transplantation, and autoimmune and inflammatory diseases (Gonzalez *et al.,* 2012).

HLA-G known as a non-classical HLA class I protein molecule, belonging from HLA family. Among extra villous

cytotrophoblasts, cornea, thymic medulla and pancreatic islets, HLA-G proteins are resident (Kovats *et al.,* 1990; Cirulli *et al.,* 2006; Ferreira *et al.,* 2017; Ribeyre *et al.,* 2018).

There are two forms of HLA-G proteins: Membrane-bond HLA-G (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and soluble isoforms, (HLA-G5, HLA-G6 and HLA-G7) as a result of alternative splicing in the primary mRNA. However, proteolytic cleavage of cell surface HLA -G1 by metallo- proteinases (MMPs) such as MMP-2 results in another soluble isoform called shedding HLA-G1 (Rizzo *et al.,* 2013).

(ILT2)/LILRB1, ILT4/LILRB2, KIR2DL4, CD8 and CD160 are receptors located on immune cells surfaces, by which HLA-G conjugated and immune-suppressive functions achieving (Fainardi *et al.,* 2011; Lin and Yan, 2019).

ILT2/LILRB1 is present on all monocytes and B lymphocytes, and on subsets of dendritic cells (DCs), myeloid derived suppressive cells (MDSCs), natural-killer (NK) cells and T cells. ILT4/ LILRB2 is expressed on DCs and monocytes, neutrophils and MDSCs (Baudhuin *et al.,* 2013; Kostlin *et al.,* 2017).

HLA-G/receptors (particularly ILT2 and ILT4) mechanisms were summarized by hindering the proliferation, differentiation, cytotoxicity, cytokine secretion and chemotaxis of immune cells, induction of regulatory cells and MDSCs or M2 type macrophages (Morandi *et al.,* 2014; Lee *et al.,* 2015).

On other hand, these proteins have a distinguished binding with immune cells by leukocyte immunoglobulin like receptor subfamily B member 4 (LILRB4). Also defined as one of LILR family member. This receptor exhibited on the surface of antigen-presenting cells (APC), such as dendritic cells and macrophages, and is also expression in vascular endothelial cells. LILRB4 structure involved two 2-type structural domains that are linkage with ligands, and cytoplasmic region concluded four immune-receptor tyrosine-based inhibitory motifs (ITIMs) (Garner *et al.,* 2006; Li *et al.,* 2019).

However, LILRB4 played a vital role in the occurrence and development of various diseases through the regulation of natural immunity and inflammatory reactions (Katz, 2007; Jiang *et al.,* 2017).

One of the major immunity molecules that able in *Leishmania* resistance and considered as a Key in immune response switching on is IL-12. However IL-12 stimuled two forms of macrophages: T-cell dependent and independent activation, Th-1 maturation and Natural killer cells (NK) efficacy to production IFN-^{3} for elevation of anti-microbial activation and induction cellular toxins of T-cells that enhance phagocytosis and reactive oxygen producing (Ismail *et al.,* 2017).

In this study aimed to investigate the alteration in sHLA-G and its receptor levels could be impressed the mean level of IL-12 in VL patients, also tried to finding any correlation between them.

Materials and Methods

Blood sample collection

Five ml of venous blood were drawn from forty-seven children samples from different Iraqi hospitals were suspected they might be infected with VL infection. The blood was placed in a sterilized plain tube and left to stand for 30 minutes at room temperature to clot and then centrifuged for ten minutes at3000 rpm for serum collection. The aspirated sera were stored in a deep freeze into sterile tubes until used. All the forty- seven samples succumbed to diagnostic tests with MHCG1, LILRB4 and IL-12 Elisa kits.

Human MHCG (Major Histocompatibility Complex Class I G) ELISA kit

The test used in detection of human HLA-G proteins. The procedure was done according to the direction of manufacture (My biosource), MHCG ELISA kit.

Leukocyte Immunoglobulin like Receptor subfamily B member 4 (LILRB4) ELISA kit

This kit target Leukocyte Immunoglobulin like Receptor subfamily B member 4 (LILRB4) which encoded by LILRB4 protein. The procedure was done according to the direction of manufacture (My biosource), LILRB4 ELISA kit.

Interleukin- 12 (IL-12) ELISA kit

This kit used for detection of IL-12 cytokines that produce from macrophage through T-cell immunity response. The procedure was done according to the direction of manufacture (My biosource), IL-12 ELISA kit.

Results and Discussion

This study included forty- seven children samples that suspected of having VL and twenty apparently healthy children as a control group. All samples were tested by rk39, MHCG1, LILRB4 and IL-12 ELISA kit.

The results showed that out of 47 patients only two patients revealed –ve result and none of healthy samples showed +ve results. However, all the positive results 45/ 47 were IgG positive, no patient with IgM appeared by rk39 study. Which means all patients were chronic

infected with VL.

All the 45 children that confirmed with visceral leishmaniasis showed a high significant (Pd≤0.01) increase in late stages (55.55%) than patients in initial stages of infection (44.44%). That is mean the patients were in chronic stage and sever infection.

VL infection has a different phases. The first one known by early or initial phase who characterize with variable duration and early symptoms. In this stage the patient suffered from many swings in the body such as, intermittent fever, malaise and shivering after special period. The firstly symptoms altered to be concluded splenomegaly, in some patients can observe hepatomegaly associated or not with splenomegaly and that absence in autoimmunod eficiency as AIDS patients (Rodrigues *et al.,* 2016). Immune complexes associated with nephritis, proteinuria, microscopic hematuria, anemia, thrombocytopenia and neutropenia (Rodrigues *et al.,* 2016; Jayakumar *et al.,* 2019).

Another phase called with late or advanced, involved thrombocytopenia, severe mucosal hemorrhage, jaundice, and ascites (accumulation of fluid in abdominal cavity) also occur (Osman *et al.,* 2001; Rodrigues *et al.,* 2016).

Highly significant increase (Pd≤0.01) were recorded in the level of sHLA-G in VL patients (17.951 ± 7.78 ng/ml) in comparison to control (0.177 ± 0.12 ng/ml). This result was agreed with previous result by Pistoia *et al.* (2007) who found higher significant increase in sHLA-G in a sera from patients affected by a variety of disorders in comparison to control group. (Fig. 1).

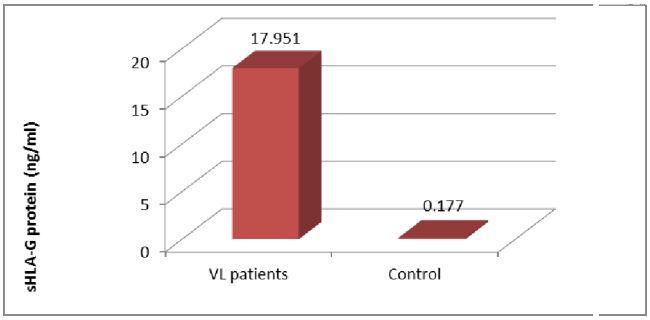

Fig. 1: Comparison between VL patients and healthy control group in HLA-G proteins.

This finding suggest that sHLA-G level may predict VL presence in suspected patients and have therefore prognostic value. However, many studied with large number of patients are needed to obtain conclusive evidence in support of this possibility.

However, the current study found the late stage recorded highly significant increase in (23.685 ± 3.82 ng/ml) sHLA-G level while (10.784 ± 4.97ng/ml) in the initial stage.

This result came in line with previous result by Morandi *et al.,* (2007) who showed the serum levels of sHLA-G were significantly higher in patients who developed a local or disseminated neuroblastoma (NB) than in those who remained in remission.

Also, this study suggested that, the up-regulation in sHLA-G expression due to increased in immunity cytokines releasing. The immune regulation to *Leishmania* depended on the type of T-lymphocyte and special cytokines as IFN-³, IL-12, 1L-10, TNF-â and etc.

In fact HLA-G may be noticed either with benefit roles as in inflammatory and autoimmune diseases or detrimental in tumors and other infections (Clement *et al.,* 2007). This study hypothized that, the soluble HLA-G showed in excess concentration in the VL patient's blood stream with dangerous roles to human's healthy.

HLA-G proteins can suppressed the immunity system through, prevention of T cell functions by foul all of T-cell mechanisms as proliferation, cytotoxicity, apoptosis and regulatory T cells. Also the differentiation, proliferation mechanisms and cytokine production in B lymphocytes (Morandi *et al.,* 2016).

The prevention of proliferation and cytotoxicity of peripheral blood NK cells, stimulated two mechanisms as proliferation and releasing of pro-angiogenic factors in blood and uterine NK cells. By down-regulating of chemokine receptors appearance on T, B and NK cells surfaces inhibits can suppress the chemotaxis of different of these cells populations, inhibition of phagocytosis process by impaired the production of reactive oxygen species in innate cells (Alvar *et al.,* 2020).

HLA-G proteins can be achieved their immunomodulatory functions through conjugate occurred between their receptors and other receptors expressed on immune cells surface. As LILRB1 (ILT2 /CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d) (Alegre *et al.,* 2014).

The expression of these receptors can be subjected to HLA-G without any costimulatory requirement as "autonomous" from any immune response (Lemaoult *et al.,* 2005), in spite of some inflammatory cytokines may be managed HLA-G expression (Ullah *et al.,* 2019).

Beside, this study recorded highly significant increase (Pd≤0.01) in LILRB4 receptor level (5.149 ± 2.043ng/ml) in VL patients in a comparison to heathy control group (0.279 ± 0.012 ng/ml). (Fig. 2).

This study suggested the LILRB4 increasing may

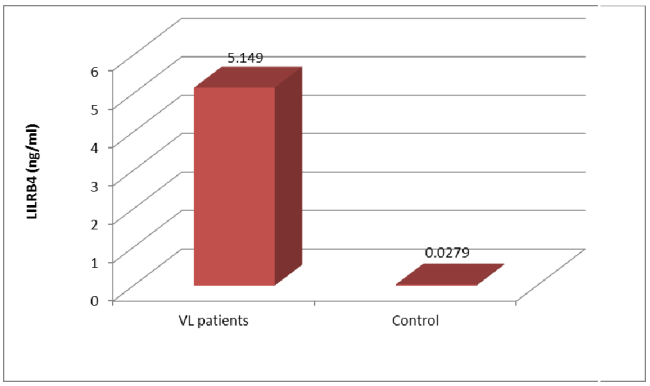

Fig. 2: Comparison between VL patients and healthy control group in LILRB4.

be related to the highly expression of sHLA-G protein that trigger the up-regulation of immunoglobulin like transcript 3 (ILT3) and (ILT4) inhibitory receptors on different immune cells especially, monocytes, macrophages and dendritic cells rendering these antigen-presenting cells (APCs) tolerogenic.

Tolerogenic APCs show reduced expression of costimulatory molecules and induce antigens - specific unresponsiveness in $CD4^+$ T-helper cells (chang *et al.,* 2002).

Tolerogenicity of professional and professional human APCs due to ILT3 and ILT4 inducing anergy and regulatory function in T-cells with cognate specificity (Vlad *et al.,* 2009). Both membrane and soluble ILT3 are proteins with potent immune-suppressive activity which are of importance for treatment of rejection, autoimmunity and cancer (Suciu-Foca and Cortesini, 2007).

However, LILRB4level was highly significantly ($Pd \leq 0.01$) decrease in initial stage of disease (3.328 ± 0.66 ng/ml) in comparison to late stage (6.969 ± 0.89 ng/ml). This study suggested that the LILRB4 up regulation knuckled under VL stress. This happened may be due to the negative feedback regulation of immunity system due to *Leishmania* impacted in *vivo* and that considered one of the deception ways to easier spreading disease. *Leishmania* parasites may be used the LILRB4 as downstream in inhibitory signaled pathways.

Also this study suggested that any change happened in HLA-G may be affected on LILRB4 concentration and role. Inhibitory LILRB4 could observe in many other cases as cancerous cells, immune cells and also in leukemia including acute myeloid leukemia (AML) (Takeda and Nakamura *et al.,* 2017).

In addition to, a highly significant ($P \leq 0.01$) increased showed in the mean level of interlukin-12(IL-12) level in VL patients (249.094 ± 79.34 pg/ml) in comparison to control (25.079 ± 3.19 pg/ml).This result was agreed with previous reports that showed a highly significant increase in IL-12 in VL patients in a comparison to healthy control group (Barral- Netto *et al.,* 1998; Khoshdel *et al.,* 2009; Andargie and Ejara, 2015). (Fig. 3).

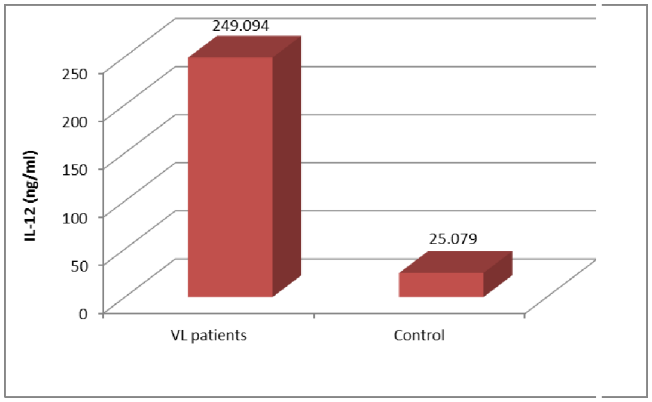

Fig. 3: Comparison between VL patients and healthy control group in IL-12.

In general, IL-12 can be observed with normal level in healthy individual. In special cases, the uncontrolling of IL-12 may be leading to several disadvantages, for instant: the over-production in IL-12 level can be injurious as in cases acute endotoxemia or autoimmune diseases. While other disordered associate with the down regulation in IL-12. Both decreasing and increasing in cytokine generation related to some alteration that intervened with IL-12 transcription genes. Wherefore, the lusty systematization of IL-12 is important for generate a special immune responses without any mistakes leaving (Cappiello *et al.,* 2020).

In vivo, the studies showed that IL-12 produced is important to control Th-2 expansion and to promote Th-1type response (Sypek *et al.,*1993; Heinzel *et al.,* 1993; Heinzel *et al.,* 1995; Murray *et al.,* 1997). Neutralization of IL-12 leads to disease exacerbation in *L. major* and *L. donovani* infections (Wang *et al.,* 1994; Heinzel *et al.,* 1995; Engwerda *et al.,* 1998).

However, this study recorded highly significant (Pd"0.01) increase in IL-12 mean level (308.20 ± 81.14 pg/ml) in a comparison to late stage of disease (195.294 ± 23.66 pg/ml). (Table 1).

The current study hypothized that any sudden modification in IL-12 may be referred to some-thing wrong occurred in *vivo* and the induction may be positively or negatively to naïve T-lymphocytic cell activation as immunity response, as in Sun *et al.,* (2015) suggestion.

Under VL conditions, the immunity response depended on the development of T helper type I immune

Table 1: The mean level of HLA-G, LILRB4 and IL-12 in VL patients in a comparison to control group.

	sHLA-G	LILRB4	IL-12
No. of control	20	20	20
No. of VL patients	45	45	45
Mean ± SD of control(ng/ml, pg/ml)	0.177 ± 0.12	0.279 ± 0.012	25.079 ± 3.19
Mean ± SD of VL (ng/ml, pg/ml)	17.951 ± 7.78	5.149 ± 2.043	249.094 ± 79.37
Mean ± SD in initial stage	10.784 ± 4.97	3.328 ± 0.66	308.20 ± 81.14
Mean ± SD in late stage	23.685 ± 3.82	6.969 ± 0.89	195.294 ± 23.66
t-test	2.641**	1.0866**	39.264 **
P- value	(0.0001)	(0.0001)	(0.0001)

**(P≤0.01)- HS.

responses (cell mediated immunity) where initiated with Interleukin-12(IL-12) production by antigen presenting cells (APCs) that induce Interferon-^{3} (IFN-^{3})-secreting (Th-1) T cells (Dayakar *et al.,* 2019).

In present study suggested there were many factors contribute in switching on immune activation and recognize there are some mistakes occurred in host body through VL infection. One of these pointers is HLA-G level alteration. HLA-G expression could be considered as one of immune- evasion strategy in the host-parasite interplay.

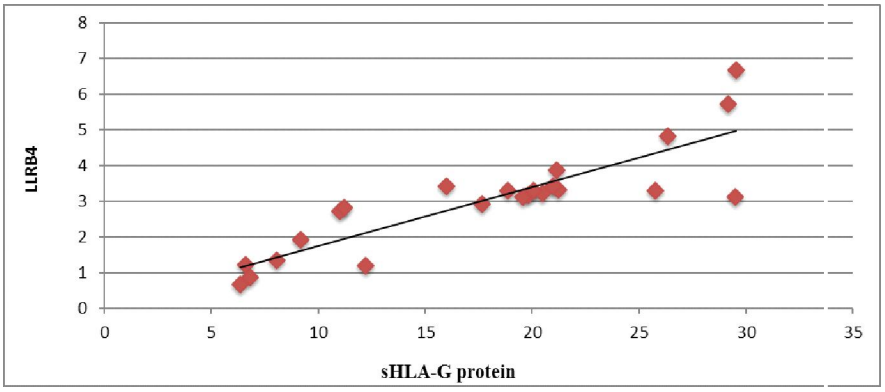

Fig. 4: Relationship between sHLA-G protein & LILRB4.

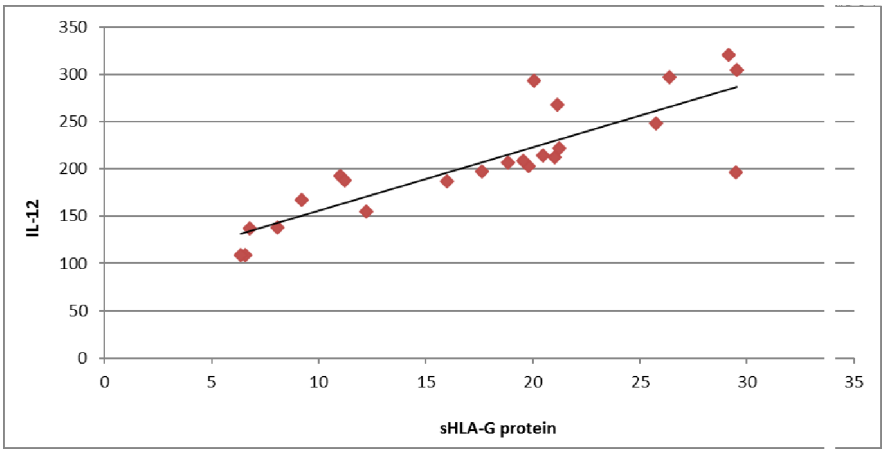

Fig. 5: Relationship between sHLA-G protein & IL-12.

In Amiot, (2014) research found soluble HLA-G level was highest in patients suffered from HIV and *Leishmania infantum* more than from HIV negative and healthy individuals.

So, similar results recorded in this study. That there were an up- regulation of sHLA-G and LILRB4 levels expressed in patients confirmed with VL infection while, a down- regulation in IL-12 was happened in VL patients with different clinical parameters, this may affected by the elevation levels of HLA-G and its receptors. According to the relationship between sHLA-G, LILRB4 and IL-12, the results showed that there was a significant linear correlation between sHLA-G and LILRB4 receptor. (Fig. 4).

Interestingly, the results assumed that there was a linear significant correlation between IL-12 and sHLA-G and LILRB4. (Fig. 5 and 6).

In other clear meaning, when sHLA-G level increased can be guess elevation in LILRB4 level. However, increase in IL-12. (Table 2).

So, there was an initial immune response to combat *Leishmania* parasites replication, but there was an immune modification happened that change this effort. Previously, all researches concentrated on the cytokine strom interaction, but the present study hypothesized that, both risk of infection and immune response may be considered as a kings of chess and the only managers are sHLA-G and IL-12 amounts in the body.

In this study also, suggested that the high levels of IL-12 in serum during

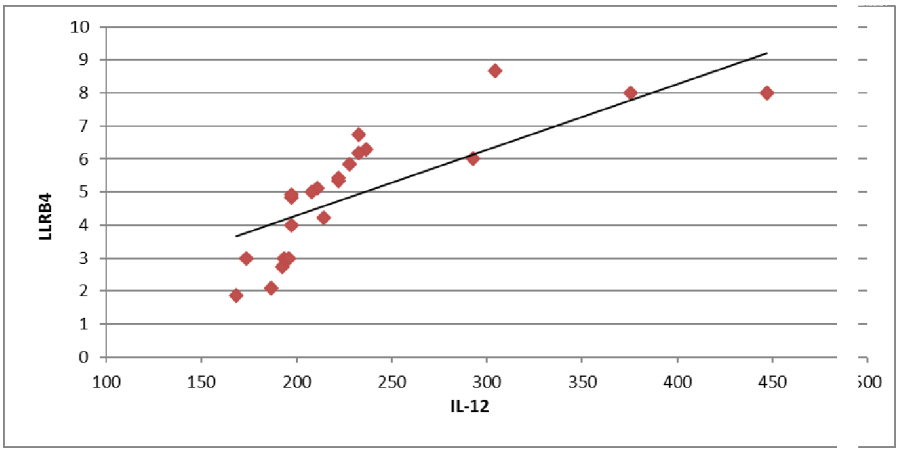

Fig. 6: Relationship between LILRB4& IL-12.

Table 2: Estimate of correlation coefficient between variables of patents in samples study.

Variables	Correlation coefficient-r	Significant level
sHLA-*G* protein & LLRB4	0.71	0.0001 **
sHLA-*G* protein & IL-12	0.88	0.0001 **
LLRB4 & IL-12	0.91	0.0001 **

** (P≤0.01)-HS.

the initial phases of infection observed in this study due to decreased in host susceptibility to VL and immunity activated yet. The highly abundance of IL-12 increased the ability of $CD4^+$ T cells to produce IFN-³. In other words, MΦ cells as yet actively and able to express more IL-12 cytokines for induced naïve T-cells after cell-cell conjugation occurred between CD40 costimulater and CD40 ligands for limited infection. Finally, a lot of naïve MΦ cells where be induced to maturation as a response to T-cells differentiation.

In VL immune regulation, the upregulation in IL-12 did not aid in maturation and differentiation only but also releasing of a variety of inflammatory substances for inflammatory effects creation (Khayrullina *et al.,* 2008).

In contrast with VL patients in chronic duration of disease, the current study suggested that the IL-12 may be declined in serum in comparison with healthy and individuals in early duration of infection. The deficiency expression of IL-12 receptors and releasing may be related to many factors as elevation in some cytokines as IL-10 and IL-4 in addition to increase in HLA-G expression.

Beside, this study agreed with Zhange, *et al.,* (2017) research, where recorded that, the HLA-G levels could be altered in cancer, auto-immune deficiency and tumors cases and he considered these proteins act as a main pointer can be dependable in diagnosis.

Also, this study suggested that, the HLA-G antigens can induce immunological tolerance especially in the late stage of VL infection by inhibit certain immune-competent cells. Because of immune-suppressive functions of HLA-G, it acts as a mediated binding of sHLA-G to private inhibitory receptors. However, HLA-G antigens may be resembled as a ligand that targeted for several immune receptors which expressed on immune cells surfaces.

When HLA-G ligands bind with specific immune cell's receptors causes switching off to MΦ and others immune cells to prevent IL-12 also releasing T-cells activation. Furthermore, HLA-G mechanism was facilitate the escaping of *Leishmania* parasites from antigen-specific immune response to safety places and as a result, the immunity system's equilibrium suffered from faults due to suitable a microenvironment creation that allowing in spread infection and parasites persistence.

Finally, this study suggested almost the host response under VL condition might be became amplified and following with dysregulation then immune suppression could be elicited, including macrophage deactivation, limited in antigen presentation, suppression in reproductive activity lymphocytes and anti-inflammatory cytokines secretion.

All above signs might be leading to an increased the chance for susceptibility to infection. Actually, all these immune changes happened at the late stage of disease. hence, there is a need for in-depth analysis of the role other cytokines rather than IL-12 and *Leishmania* pathogenesis to get a comprehensive view of the complex interplay of *Leishmania* parasite and their host, keeping in mind Th1/Th2 balance is not the only determinant of the outcome of leishmaniasis as previously thought due to the high expression of HLA-G in VL patients that may implicated in disease progression.

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