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Hepcidin expression by THP-1cells upon stimulation with Pam2 via TLR2/6

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Abstract
Hepcidin plays a central role in orchestration on iron metabolism also providing a link between iron metabolism, inflammation and innate immunity. In this present study, we investigated the intrinsic ability of THP-1cell which have been differentiated to macrophages to expressed hepcidin in-vitro and involvement of pattern recognition by toll-like receptor (TLR6/2) stimulated with synthetic Pam2. This present study indicates that the binding of Pam2 and TLR2/6 directly triggers hepcidin expression in macrophages. This shows that hepcidin can be produced in the local environment of bacterial infection, therefore playing an essential role in the innate immune system. This is important because the pathogen producing the inflammation can be killed quickly via iron withdrawal, without inflammatory cytokines having to reach the liver first. These result indicates that the iron regulatory hormone and antimicrobial peptide, hepcidin, can potentially represent a host defence mechanism against intracellular pathogens and provides hope for new chemotherapies against intracellular diseases. There is need for further studies to investigate the potential development of new chemotherapies that takes into consideration the potential antimicrobial effect of the peptide, hepcidin in the management of intracellular pathogens.

1. Introduction

Iron is a vital nutrient for virtually all forms of life. The requirement for iron is based on its role in cellular processes ranging from energy generation, respiration, the tricarboxylic acid cycle, lipid metabolism, gene regulation and DNA replication to oxygen transport and protection against oxidative stress (1). Bacterial pathogens are not exempt from this iron requirement, as these organisms must acquire iron within their vertebrate hosts in order to replicate and cause disease (2) during infections, pathogens use various means to acquire iron from their hosts, while hosts attempt to withhold it from pathogens (3). Iron therefore represents a point of conflict between host and pathogen, and altered iron balance associates with poor outcomes in several infectious diseases, including malaria, salmonellosis (4) tuberculosis (5) and Human Immunodeficiency Virus(HIV-1) infection (6).

THP1 reporter cells are derived from THP-1, a human monocytic cell line that naturally expresses many pattern-recognition receptors, including Toll-like receptors. Toll-like receptors (TLRs) are pattern recognition receptors that

recognize a wide variety of microbial molecules. The ligands for these receptors are components of pathogenic microbes and are often called "pathogen-associated molecular patterns" (PAMPs) (7). TLRs are frequently referred to as a system of non-specific or "innate" immune defense, i.e, the response is present and unchanging during the life of the organism and occurs in the same manner with every exposure to the pathogen (8).

Currently much research and studies being carried out in order to define the role of hepcidin as a mediator of iron homeostasis and innate immunity but, it is not known if activation of other TLR receptors can result in hepcidin expression using synthetic lipoprotein. In this present study, we investigated the intrinsic ability of THP-1cell which have been differentiated to macrophages to expressed hepcidin in-vitro and involvement of pattern recognition by toll-like receptor (TLR6/2) stimulated with synthetic PAM2.

2. Materials and Method

Informed consent in writing was obtained from each subject participating in the study, and the study protocol conformed to the ethical guidelines of the Cardiff Metropolitan University and was approved by the review School Ethics Committee at the Cardiff Metropolitan University UK.

2.1. Cell Culture Media and Reagents

Monocyte and Macrophages are one of the most important cellular components of the innate immune System (9). These myeloid cells expresses a variety of Pattern Recognition Receptor that allowed them to recognized and response to microbial component contributing to the elimination of invading microorganisms (9). Cell line THP-1(EATCC TIB202), originally isolated from a child with acute leukemia, and are matured cells in the monocyte/macrophage lineage with a normal diploid karyotype (10), and they produce TNF-a and other cytokines in response to purified endotoxin (11). The cell has characteristic of human monocytes. THP-1cell has ability to carry out phagocytosis and produce lysozyme and cytokines such as TNF-α. Furthermore, they express surface Fc and C3b receptor, but no surface or cytoplasmic immunoglobuling.

2.1.1. Reagents

The human THP-1monocytic cell line were obtained from European cell culture collection and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium were obtained from Life technologies UK, supplemented with stable Glutamine 2Mm, Sodium pyruvate 1Mm, non-essential amino acid (IX) and phosphate buffered saline (PBS) all were obtained from PAA Cell culture Austria and 10% heat-inactivated Foetal Bovine Serum(FBS) low endotoxin treated were obtained from Laboratory technology International UK and 20mM of PMA stock solution, Forward & Reverse hepcidin primers and GABDH was all obtained from Sigma Aldich dorset, UK. Pam2CSK4 is a synthetic diacylated lipopeptide was purchased from Invivogen. M-MLV Reverse Transcriptase, first strand buffer, dNTPs, Taq polymerase/ Supermix, Ramdom pimers and Trizol were all obtained from invitrogen/Life technology. Chloroform, isopropanol and ethanol were purchased from *Fisher Scientific* UK – *Loughborough.*

2.2. Culture of THP-1cell

The human monocytic cell line THP-1was purchased from the European Collection of Animal Cell Culture (ECACC). Cells were grown in complete medium consisting of RPMI (Roswell Park Memorial Institute) 1640 medium (Life technology, UK), supplemented with 10% v/v foetal bovine serum (FBS; Laboratory technology internation, UK), 2mM of stable glutamine, 1% non-essential amino acids, and 1Mm of sodium pyruvate all was purchased from (PAA cell culture). Cultures were maintained in 25cm^3 tissue culture flasks (Greiner-Bio One, Germany) and incubated at 37^o C in a humidified 5% CO_2 incubator. The cells culture suspension were grown to about 80% density and used for the experiments or were sub-cultured for further use. The cells were passaged weekly to maintain exponential cell growth. Cell viability and cell counting used the Trypan Blue staining method.

Cell enumeration were carried out to ascertain the maximum value at which the cell were maintained i.e 1×10^6 cells/ml and the cells was further centrifuge to replace the fresh adjusted volume to double what is required i.e 2×10^6 cells /ml. 2 x 106 cells/ml in complete medium, these were dispensed in 24-well tissue culture plate (2 ml per well).

2.2.1. Propagation

These cells were maintained at a density of between $1x10^6$ cells/ml to $1x10^6$ cells/ml ($2x10^6$ cells/ml maximum). If the cells were allowed to propagate more, it gets denser, and then this may slow down or stop dividing and may clump or have an irregular or blebbing appearance.

2.2.2. PMA Preparation

10 mM of stock solution of (PMA: Sigma Aldich dorset, UK) was prepared DMSO Aliquoted in endotoxin free-vial (10µl/vial) and stored at -70^{O}c until use. Thp1-cell was treatment with phorbol esters, to allowed the cell to differentiate into cell with similar characteristic to that of the human macrophages expressing adhesion molecules that allow them to form monolayer on the surface of plastic tissue culture flask (T-25 tissue culture flask)

2.2.3. Synthetic Pam2 Stimulation

Pam2CSK4 is a synthetic diacylated lipopeptide (LP). According to the current model, diacylated LPs induce signaling through TLR2/6 (12) PAM2 was used as Ligand during the research work for the determination of hepcidin

expression and pro-inflammatory cytokine such as TNF-α

2.3. Cell Stimulation

Undifferentiated THP-1cells (1×10^6 cells/ml in medium containing 10% FCS) were incubated in 24-well plates (2ml/well) with PAM2 (invivogen) at different concentrations 100ng/ml, 75ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, 3.125ng/ml and PMA-Differentiated Thp1-cell (1 × 106cells/ml in medium containing 10% FCS) were incubated in 24-well plates (2ml/well) with PAM2 (invivogen) (50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, 3.12ng/ml, 1.56ng/ml, 0.78ng/ml) for the time points indicated in the corresponding figures 1 and 2. In control experiments pam2 was replaced with the corresponding volume of thp1-cell (media). Both Differentiated and undifferentiated Thp1 cell were analysed along with control in triplicate which contained only the media. Each of the concentration above was run in triplicate in 24 well titre plates. After incubation, the Aliquots of supernatant were harvested into 1ml of Ependorf tube and freeze over night for ELISA on the next day (DuoSet ELISA; R and D). Cell pellets were lyzed with TRIzol® Reagent (Invitrogen) and used for RNA isolation.

2.4. Enzyme Linked Immunosorbent Assay

The supernatants obtained from the stimulation experiment, were analysed for TNF-α content by ELISA (DuoSet ELISA; R and D) following the manufacturer instructions in 96-well ELISA plates (Nunc-Immuno Maxisorp, Denmark). Briefly, the wells on the ELISA plate were coated overnight with 100 µL of TNF-α coating antibody (diluted in phosphate buffer saline/PBS), sealed and incubated overnight at room temperature. The plate was then washed out 3 times with ELISA washing buffer (PBS supplemented with 0.05% Tween-20) and blocked with 300 µL/well of blocking buffer (1% bovine serum albumin/BSA in PBS). Plates were sealed and incubated for 1 hour at room temperature. The wells were then washed 3 times with ELISA washing buffer and then 100 µL of either sample or standards diluted in reagent diluent (Tris buffer supplemented with 0.1% w/v BSA) were applied to the appropriate wells. Plates were sealed and incubated at room temperature for 2 hours. The washing step was repeated and then 100 µL of biotinlylated anti-TNF-α detecting antibody was added to each well, the plate was sealed and incubated at room temperature for 2 hours. The washing step was repeated again, and 100 µL of Streptavidin-HRP at a dilution of 1/200 in reagent diluent were added to each well and incubated for 20 minutes at room temperature. The washing step was repeated again, and 100 µL of substrate Sure Blue TM TMB (Kirkegaard & Perry Laboratories, Inc.) were added to each well and the plate placed in a dark place and incubated for approximately 15 minutes. Once the colour was fully developed in the standards, 50 µL of 1M HCl were added to each well to stop the reaction. The results were then read in an ELISA reader (Tecan, UK) which determined the optical density at 450 nm as an indirect measure of TNF-α concentration in the samples.

3. Result

3.1. Hepcidin Expression by THP-1 Cells before and after Differentiation

Hepcidin Expression by THP-1Cells stimulated with synthetic Pam2 was the main objective of the present work to test the capacity of THP-1monocytic cells line to express hepcidin mRNA constitutively or upon Stimulated with Synthetic Pam2. To this aim, both differentiated and undifferentiated THP-1cells were treated with 4 different concentrations of Pam2 (1ng/15ml and 0.5ng/15ml) for undifferentiated Thp1-cell and (1ng/6ml and 0.5ng/6ml) for differentiated Thp1-cell. In untreated control cultures Pam2 was replaced with cell only, After 48 hours (data not show), cells were collected and hepcidin mRNA expression analyzed by RT-PCR. Table 1 shows the dilution table for PAM2 and undifferentiated THP-1 cell.

3.2. Undifferentiated THP1-Cell Does Not Expressed Hepcidin upon PAM Stimulation

Results presented in Figure 1 shows that under the culture condition used, THP-1cells (undifferentiated) constitutively does not expressed hepcidin mRNA as the corresponding 98bp and 250bp band was clearly not observed. Treatment with (1ng/15ml and 0.5ng/15ml) Pam2 does not altered the basal expression of hepcidin in immature Thp1-cell. However, a significant expression of housekeeping gene (GAPDH) was observed, at 500bp suggesting that, the experiment has worked but undifferentiated Thp1 cell does not expressed hepcidin even though when the cell were treated with high doses of Pam2 concentration. Table 2, 3 and 4 show the absorbance of undifferentiated THP -1 cell using PAM2 for stimulation to produced TNF alpha, protocol of ELISA graph for undifferentiated THP1-cell and protocol of ELISA graph for PMA- differentiated THP1-cell respectively.

3.3. RT-PCR- RNA Extraction

Total RNA was extracted using TRIzo®(Invitrogen/Lifetechnologies) RNA Isolation Reagents according to the manufacturer's instructions after the addition of pam2 at concentration of 1ng/15ml & 0.5ng/15ml to undifferentiated and 1ng/6ml & 0.5ng/6ml to PMA- differentiated Thp-1cell. RNA was isolated from THP-1, after the disruption and homogenization of human monocytic cell line.

3.4. RNA Extraction

Table 1. *Dilution table for PAM2 and undifferentiated THP-1 cell*

Working stock volume (ml)	Media Volume (ml)	Concentration in Ng
5.0	0.0	100
3.5	1.5	75
5.0	5.0	50
5.0	5.0	25
5.0	5.0	12.5
5.0	5.0	6.25
5.0	5.0	3.125
5.0	5.0	1.562
0	0	0

RNA was obtained by harvesting the THP-1cells (undifferentiated and PMA-differentiated) of at least 10^6 cells in 5ml of media. The cell were in triplicates and was pooled into a single tube and treating each cell pellet with 0.5 mL of TRIzol (Invitrogen/Life technologies). After 5 minutes incubation at room temperature, 200 µL of chloroform (Fisher Scientific Loughborough) were added, mixed well (approx. 15 second) and vials kept at room temperature for 3 minutes. The samples were then centrifuged in a pre-cooled micro-centrifuge at 4 °C for 15 minutes at 12,000 RCF. Following centrifugation, the upper aqueous phase was transferred into new tube and RNA was precipitated by adding 500 µL of isopropanol (Fisher scientific Loughborough). After 10 minutes incubation, the centrifugation was repeated under the same conditions. Then, the supernatants were discarded and the pellets (RNA) washed with 1 mL of ice-cold 75% (v/v) ethanol/water (Fisher Scientific Loughborough) and centrifuged at 4 °C for 5 minutes at 7,500 RCF. The washing procedure was repeated twice. Finally, the ethanol was removed and the pellets left to dry for approximately 45 minutes. The RNA pellets were dissolved in 50 µL of water (molecular biology water) and used immediately for preparation of cDNA or stored at -80 °C. Table 5 and 6 show the quantification of RNA using Nano drop software and to determine the quality of RNA extracted in undifferentiated and differentiated THP1-cells respectively.

Table 2. *Absorbance of undifferentiated THP -1 cell using PAM2 for stimulation to produced TNF alpha*

Conc. of pam2	OD of TNF-α, SAM 1	OD of TNF-α, SAM 2	OD of TNF-α, SAM 3	Conc. of TNF-alpha	SD
100ng/ml	0.2101	0.0833	0.0769	0.123433333	0.07512372
75ng/ml	0.1372	0.1804	0.0659	0.127833333	0.057821824
50ng/ml	0.0699	0.069	0.0536	0.064166667	0.009162059
25ng/ml	0.064	0.0522	0.0509	0.0557	0.00721734
12.5ng/ml	0.0454	0.0598	0.0538	0.053	0.007233257
6.25ng/ml	0.0454	0.0379	0.049	0.0441	0.005663038
3.125ng/ml	0.0492	0.0453	0.0435	0.046	0.00291376
Control	0.0201	0.0204	0.0208	0.020433333	0.000351188

Table 3. *Protocol of ELISA graph for undifferentiated THP1-cell*

Conc. of PAM2	Conc. of TNF-alpha	Cell only/Control
Control	0	0.0204
3.125ng/ml	0.046	0
6.25ng/ml	0.0441	0
12.5ng/ml	0.053	0
25ng/ml	0.0557	0
50ng/ml	0.064166667	0
75ng/ml	0.127833333	0
100ng/ml	0.123433333	0

Table 4. *Protocol of ELISA graph for PMA- differentiated THP1-cell*

Conc. of PAM2	Conc. of TNF-alpha	Cell only/ control
Control	0	0.0565
0.781ng/ml	0.661366667	0
1.562ng/ml	0.668733333	0
3.125ng/ml	0.637966667	0
6.25ng/ml	0.6944	0
12.5ng/ml	1.052	0
25.5ng/ml	0.890566667	0
50ng/ml	1.070466667	0

Table 5. *Quantification of RNA using Nano drop software and to determine the quality of RNA extracted in undifferentiated THP1-cell*

S/No	Sample Number	Concentration in ng/µl	Absorbance value At 260/280nm
1	7	60.5 ng/µl	1.74-0.60
2	8	39.0 ng/µl	1.84-0.68
3	9	49.1 ng/µl	1.87-0.70
4	10	84.9 ng/µl	1.81-0.39
5	11	75.8 ng/µl	1.89-0.46
6	12	26.2 ng/µl	1.81-0.60
Control cells only			
7	1	38.1 ng/µl	1.72-1.46
8	2	48.4 ng/µl	1.80-1.11
9	3	77.6 ng/µl	1.63-0.70

Lane A: A commercial ladder containing fragment of different sizes which measure the base pair of gene expression as indicated in the figure above.

Lane B: Serve as the control in the experiment, it contain cell only (thp1-cell)

Lane C: Contain the higher concentration of Synthetic Pam2 used for the stimulation of undifferentiated Thp1-cell.

Lane D: is the reference housekeeping gene used in the

experiment that serves as the internal control. They are expressed in all cells of an organism under normal and patho-physiological conditions (13). However, some housekeeping genes (such as GAPDH, HSP90, and β-actin) are expressed at relatively constant levels in most non-pathological situations (14).

Lane E: Same as Lane B

Lane F: Contain the lower concentration of Synthetic Pam2 used for the stimulation of undifferentiated THP1-cell

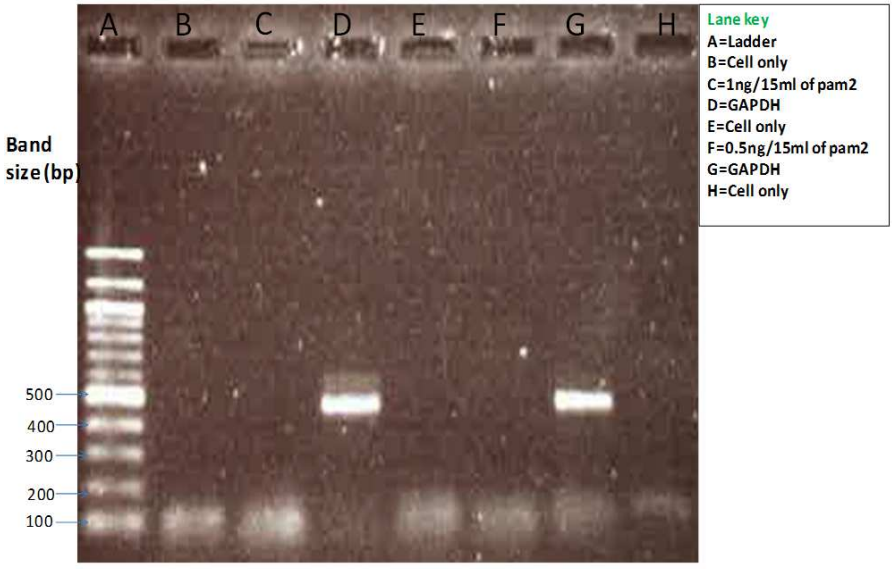
Lane G: Same as Lane D

Lane H: Serve as PCR negative control which contained distilled water (DH_2O) only

Densitometric analysis shows no hepcidin expression in undifferentiated Thp1-cell when treated with pam2 concentration of 1ng/15ml and 0.5ng/15ml. These results suggest that at the time point selected, the both concentration of pam2 used has no effect in up regulation of hepcidin mRNA expression.

Table 6. *Quantification of RNA using Nano drop software and to determine the quality of RNA extracted in PMA-differentiated THP1-cell*

S/No	Sample Number	Concentration in ng/µl	Absorbance value At 260/280nm
1	7	165.2 ng/µl	1.86-1.06
2	8	172.2 ng/µl	1.80-1.16
3	9	171.5 ng/µl	1.82-0.85
4	10	98.9ng/µl	1.86-1.34
5	11	156.7 ng/µl	1.76-0.66
6	12	124.4 ng/µl	1.80-1.75
Control cells only			
7	1	138.1 ng/µl	1.72-1.46
8	2	148.4 ng/µl	1.70-1.11
9	3	177.6 ng/µl	1.83-0.70



This figure Show no Hepcidin Expression in undifferentiated THP-1 Cell Treated With synthetic Pam2. THP-1 cells (10^6 cells per well) were cultured in RPMI medium containing 10% FBS and stimulated with 1ng/15ml and 0.5ng/15ml or Cell only (control). After 48 hours (data not shown), cells were collected, RNA isolated and hepcidin expression analysed by RT-PCR. Fragments were analysed by electrophoresis using a 2% agarose gel.

Figure 1. *Undifferentiated THP1-cell does not expressed hepcidin upon PAM 2*

3.5. PMA-differentiated THP1-Cell Expressed Hepcidin mRNA upon PAM2 Stimulation

However, the result presented in Figure 2 shows that PMA- differentiated cell significantly expressed hepcidin mRNA, which were correspond to 250bp band used when the cells were treated with increase concentration of Pam2 at 1ng/6ml and 0.5ng/6ml. It should be noted that no changes in the housekeeping gene GAPDH were observed after increased in pam2 concentration for stimulation.

Lane A: A commercial ladder containing fragment of different sizes which measure the base pair of gene expression as indicated in the figure above.

Lane B: Serve as the control in the experiment, it contain cell only /GAPDH

Lane C: is the reference housekeeping gene used in the experiment that serves as the internal control. They are expressed in all cells of an organism under normal and patho-physiological conditions (13). However, some housekeeping genes (such as GAPDH, HSP90, and β-actin) are expressed at relatively constant levels in most non-pathological situations (14).

Lane D: Contain the Lower concentration of Synthetic Pam2 used for the stimulation of differentiated Thp1-cell

Lane E: Same as Lane C

Lane F: Contain the Higher concentration of Synthetic Pam2 used for the stimulation of differentiated Thp1-cell

Lane G: Same as Lane C

Lane H: Serve as the control in the experiment, it contain cell only (thp1-cell)

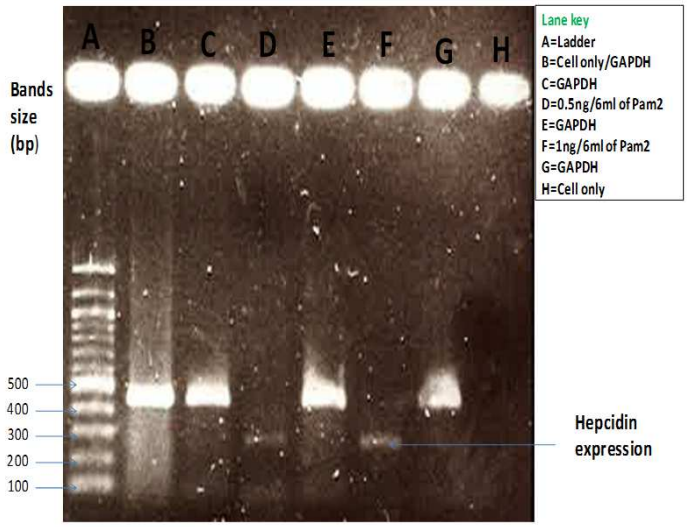


Figure 2. *PMA-differentiated THP1-cell expressed hepcidin mRNA upon PAM2 stimulation*

Densitometric analysis shows a reduction on the intensity of the hepcidin band of approximately 40% when Pam2 was used at 0.5ng/6ml and of approximately 60% when Pam2 was used at 1ng/6ml. These results suggested that at the time point selected, the intermediate concentration of pam2 used has the strongest effect up regulating hepcidin mRNA. Further work will be needed to optimised hepcidin mRNA expression under the time/duration, specification and using higher concentrations of Pam2 may be more efficient in expressing hepcidin mRNA. However No changes were observed in the housekeeping gene GAPDH.

4. Discussion

Prior to this study, it was acknowledged that hepcidin levels in the circulation are mainly determined by its production and release by hepatocytes. However, other cells of the immune system such as monocytes, macrophages and lymphocytes (15) as well as other tissues like the pancreas (16) and the stomach (17) can also express hepcidin.The contribution of these alternative sources to the overall levels of systemic hepcidin is presently unknown, but it is believed that the production of hepcidin by these cells is low and therefore only capable of affecting the local microenvironment, influencing localized responses and regulating iron levels at the site of infection (18).

Hepcidin is produced rapidly by the liver in mice in response to inflammatory stimuli, LPS, Freund adjuvant, and turpentine (19-20). Hepcidin is also produced by the liver following in vivo iron loading and anaemia and hypoxia lead to a decrease in production (21). Several studies have demonstrated that in vitro expression of hepcidin mRNA is induced by IL-6 and LPS in human and mouse hepatocytes, and by IL-1 in mouse hepatocytes (22). In vivo, liver hepcidin mRNA is up-regulated in response to inflammation resulting from the injection of mice with LPS, turpentine, and Freunds complete adjuvant (21). Several recent studies have shown that hepcidin mRNA is also expressed in mouse macrophages in response to LPS (23) infection with extracellular pathogens (24), or infection with intracellular pathogens (25).

Other studies have shown that, lipoproteins (LPP) purified from several pathogens, including *S. aureus*, as well as the synthetic lipopeptides $Pam_3CysSerLys_4$ (Pam_3Cys) (26) and $Pam_2CysSerLys_4$ (Pam_2Cys), mirroring the tri- and diacylated LPP of bacteria, are known to activate TLR2 (27-28). The three lipid chains of Pam_3Cys mediate the heterodimerization of the TLR1 and TLR2 receptor (29). Diacylated LPP signal by TLR2/6 dimers, and crystallization modeling predicted binding of lipid chains to TLR2 and stabilization by TLR6 (29). Activation of TLR2 by synthetic lipopeptides leads to induction of cytokines, chemokines, adhesins, and nitrite in macrophages and epithelial cells. The complexity of TLR2 in recognition becomes obvious since staphylococcal lipoteichoic acids (LTA) have been shown to trigger the immune response (30). However, LPP rather than LTA seem to be the dominant TLR2 stimuli in *S. aureus* strains (31-32).

According to the current model, triacylated LP like Pam3CSK4, are recognized by TLR2/1, whereas diacylated LP, such as FSL-1, induce signaling throughTLR2/6. However, it was reported that diacylated LP, such as Pam2CSK4, induce signaling in a TLR6-independent manner (33). The part of pam2 responsible for biological

activity is the N-terminal lipopeptide moiety, structure to be *S*-(2,3-bispalmitoyloxypropyl)Cys-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Ly (Pam_2CGNNDESNISFKEK). Furthermore, Inactivation of LPP maturation in *S. aureus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, and *Bacillus subtilis* resulted in reduced growth under stress conditions or in the presence of phagocytes in vitro (34-35).

In order for hepcidin to be released from macrophages, the bacteria must first be recognised as non-self. This occurs when the bacteria binds to a receptor on the surface of a macrophage. Bacteria have specific structural patterns called Pathogen-Associated Molecular Patterns (PAMPs). In this study, the PAMP of the bacteria is Pam2CSK4. These Pam2CSK4 are recognised by Pattern Recognition Receptors (PRRs) which is present in TLR. Each Pam2CSK4 binds to a specific PRR – Pam2CSK4 binds to toll like Receptor- TLR-6/2 on macrophages. This binding triggers hepcidin expression from the cell and is thought to improve the host response to pathogens, both through its direct antimicrobial properties as well as through limiting the availability of iron (36).

On the other hand, the activation of NF-κB by the purified lipoproteins of *M. fermentans* (*Pam_2*CGDPKHPKSFTGWVA) was mediated through TLR2 and TLR6, but not TLR1 (37). These results indicate that *M. fermentans* LAMPs (*Pam_2*CGDPKHPKSFTGWVA) may contain several active components, one of which is recognized by TLR2 and TLR6, while other components such as (Pam3CSK4) might be recognized by TLR1 and TLR2.

Several investigators have demonstrated that Anti Microbial Peptide (AMP) expression can be modulated by LPS (38). This important glycolipid has many effects on the immune system including its capacity to increase macrophage differentiation and phagocytosis and to suppress pro-inflammatory responses (39). However, little or none is known about the capacity of Synthetic Pam2 to regulate hepcidin expression. The present work has addressed this possibility by looking at the effect of synthetic pam2 on hepcidin mRNA expression on matured monocytic cells line. Initial experiments were tested on immature THP-1monocytic cells line which constitutively does not express hepcidin mRNA

The results presented here support this finding, as hepcidin mRNA was clearly expressed by quantitative RT-PCR of particular relevance to the work. It has also been demonstrated that lipoprotein is a direct inducer of the Hepcidin in human monocytes, keratinocytes, and neutrophils (40), suggesting a potential effect of Pam2 which is an analogy of lipoprotein on modulating the expression of other AMPs including hepcidin. Showing that the presented result support these finding. However, contrary to what was observed with cathelicidin, 1, 25 (OH) 2D3 downregulated hepcidin mRNA expression suggesting that cathelicidin and hepcidin are regulated differently by 1, 25 (OH) 2D3 (40). There is paucity of data addressing the question whether a host response in systemic infection is mediated by staphylococcal LPP (analogy of pam2) and by TLR2. However, using $TLR2^{-/-}$ mice, it has been shown that recognition of Lipoprotein by TLR2 allows inflammation, limits bacterial growth, and improves recovery of weight in TLR2 -infected mice (37). However, mouse with deficient $TLR2^{-/-}$ was completely infected and highly vulnerable. In agreement with Takeuchi and colleagues (41) suggesting that, TLR2 play an important role in the activation of innate immunity by recognizing specific patterns of microbial components as seen in the present work.

Previous studies have demonstrated the critical role of TLR2 and MyD88 against extracellular Gram-positive bacteria using knockout mice. Both TLR2- and MyD88-deficient mice developed higher mortality compared with wild-type mice after inoculation with *S. aureus (42) s*uggesting that, MyD88 is essential for the induction of inflammatory cytokines triggered by all TLRs. TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathway.

Bacterial induction of hepcidin expression in mouse macrophages and neutrophils following infection with *P. aeruginosa* and group A *Streptococcus* has been reported recently by Peyssonnaux et al (24). Other studies showed that LPS induced hepcidin mRNA in mouse macrophages (43). However, these study focused on the intrinsic ability of THP-1cell (undifferentiated & differentiated) to produce hepcidin in vitro and involvement of pattern recognition by toll-like receptor 2 (TLR2) using synthetic PAM2 for induction. Here, we reported that hepcidin is also produced in human macrophages transfected with invitro Pam2. Suggesting that hepcidin is highly inflammatory.

Defensins have been widely studied as an antimicrobial peptide family present in airway fluid and reported to possess antimicrobial activities, including those against mycobacterial infection (44). Additionally, hepcidin, an antimicrobial peptide that regulates iron homeostasis, inhibits *M. tuberculosis* growth *in vitro* and inflicts structural damage on this notorious pathogen (25). Moreover, these antimicrobial peptide molecules influence a variety of physiological processes and also function as crucial signaling mediators in host defense and inflammation (45).

The present work has demonstrated the maximal effect of hepcidin mRNA expression, which were observed when Pam2 at higher and lower concentration was used on PMA-Differentiated THP1-cell with a reduction on the intensity of the hepcidin band from 60% to 40% respectively. However, using undifferentiated THP1-cell at different concentration of pam2 resulted in complete down regulation of hepcidin mRNA expression. Our finding is in agreement with those of other investigators and indicate that an optimal concentration of Pam2 and differentiation of Thp1-cell from monocytic to macrophage is required for hepcidin expression. There is need for more work to

elucidate the threshold for maximum effect of Pam2 on hepcidin expression. It has been also reported that *M. fermentans*-derived lipopeptide MALP-2 is a potent macrophage activator (46).

Our study has shown that hepcidin can be produced in the local environment of the bacterial infection, therefore playing an essential role in the innate immune system. This is important because the pathogen producing the inflammation can be killed quickly via iron withdrawal, without inflammatory cytokines having to reach the liver first. These result supported by finding in a previous report (47) which say the iron regulatory hormone and antimicrobial peptide, hepcidin, likely represents a host defence mechanism against intracellular pathogens such as *M. tuberculosis*, and provides hopes for new chemotherapies against tuberculosis. However, the undesired consequence of elevated hepcidin levels in the circulation is the development of anaemia -AI/ACD (48). Furthermore, well-designed clinical studies addressing safety and long-term efficacy are still needed in order to clarify the risks and benefits of hepcidin-targeted treatments.

In summary, the binding of Pam2 and TLR2/6 directly triggers hepcidin expression in macrophages. There is need for further studies to investigate the potential development of new chemotherapies that takes into consideration the potential antimicrobial effect of the peptide, hepcidin in the management of intracellular pathogens like *M. tuberculosis.*

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