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Neutrophil gelatinase associated lipocalin a proinflammatory polypeptide necessary for host cell survival in bacterial infection

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ABSTRACT

During sepsis along with the manifestation of symptoms, there are many biochemical and physiological changes in the host. To mark a few are, Presence of bacteria, changes in overall proteomics of blood, which is termed acute phase response and organ failure leading to death in extreme cases. In search of biomarkers during early bacterial infection, we could identify a prominent biomarker Neutrophil gelatinase-associated lipocalin (NGAL) and its involvement in bacterial infection. NGAL is a 25 kDa protein secreted by neutrophils, whose importance is well studied in kidney injury and cancer, whereas its role during bacterial infection is less sorted. To evaluate its role, human whole blood cells (HWBC) induced with bacterial *E. coli* LPS were analyzed for changes in NGAL expression. NGAL transcripts were observed to be upregulated very early, <4 h of induction and continued to be detected until 18 h, after which its levels rapidly decreased by 24 h. Treatment of HWBC with steroid hormones such as estrogen (β Estradiol), testosterone, progesterone, and adrenaline confirmed the mode of action of NGAL as pro-inflammatory. Further, immunoblotting of lysates from HWBC treated with retinol and LPS revealed that upregulation of NGAL in the presence of retinol was reversed on induction with LPS. Organic extracts from the root of the plant *Desmotrichum fimbriatum* (DF) were explored for their effect on bacterial infection by *E. coli* (ATCC25922) and effect on NGAL. HWBC pre-treated with DF and induced with *E. coli* inhibited bacterial growth and multiplication combined with elevated NGAL and protected cells from undergoing apoptosis. This demonstrates the role of NGAL, suggesting its necessity in the host to mount antibacterial action and aid host survival. Accordingly, NGAL can be categorized as an acute phase pro-inflammatory polypeptide necessary for establishing an early immune response toward invading bacterial infection by exerting antibacterial activity necessary for cell survival.

1. INTRODUCTION

Bacterial infection leading to sepsis is a predominant cause of mortality among patients admitted to the ICU [1]. It involves many physiological changes including activation of cytokine storm, bacteremia, septicemia, and also serious malfunction of visceral organs which may rapidly progress toward mortality of the patient [2]. These consequences depend on the regulation of plasma proteins responding to the infection [3] and are generally regarded as biomarkers of sepsis [4] which can ascertain a patient's immune response and can be broad predictors of severity [5] for devising appropriate therapeutic strategies [6]. The hunt for new biomarkers to predict infection and risk of morbidity is still pursued assessing the immune status of patients as several known biomarkers fail to predict the immune status and risk of morbidity [7]. For example, CRP and Procalcitonin have established markers commercially utilized to diagnose infection and sepsis but cannot predict the risk of morbidity [8]. In this context, a small glycoprotein called neutrophil gelatinase-associated lipocalin (also called NGAL, lipocalin 2, and siderocalin) was explored to understand its role in bacterial infection and underline its importance as an early biomarker of inflammation [9]. It is known that NGAL is considered a kidney failure biomarker far superior to creatinine [10] since it is found in the serum of injured kidney patients at the onset of renal injury [11,12]. Although its physiological role in kidney injury is unclear, its elevation is prominent in predicting the severity of injury [13].

Several reports indicate the elevation of NGAL in sepsis by several folds, underlining its prominence as a biomarker of sepsis [14]. Hence, the present study deals with understanding the role of NGAL in bacterial infection [15]. NGAL is necessary during the early stages of infection and hence deciphering its mode of action against bacterial infection was explored [16,17].

NGAL is a 25 kDa glycoprotein carrying unique 8 anti-parallel beta-sheets known as a lipocalin fold which makes it an ideal carrier for small lipophilic molecules [18,19]. Being small and glycosylated, it can be

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easily exocytosis on infection by neutrophil granules where it is stored and circulated in blood [20]. It occurs in three isoforms - a monomer of 25 KDa, a dimer of 45 KDa, and a heterodimer of 135 KDa complexed with Metalloproteinase-9 [21,22]. Many functions have been added to NGAL being anti-tumor [23] in some cases, a few cases promoting angiogenesis, its implication in cardiovascular diseases [24,25] and also during inflammation [23].

Antibacterial activity of NGAL and its upregulation during bacterial infection can have therapeutic applications in controlling the spread of diseases by inhibiting bacterial growth [26,27]. It sequesters available iron during a depleted iron environment and exhibits antibacterial activity and hence boosting innate immunity [15]. Apart from being antibacterial, its implication in inflammation is also well studied [28,29]. In this study, changes in NGAL levels in response to inflammatory conditions and infection were studied by comparing with known biomarkers such as IL6, TNFα, and Procalcitonin [30,31]. After upregulation during defined conditions, analysis fate of NGAL plays a crucial role in underlining its impact on constructive or destructive regulation in the host.

In our study, we employed simulation studies to explore the relationship between NGAL and bacterial infection and its role in modulating the inflammation signals. Early expression of any molecule during infection and its detection provides better insight for treating a patient in critical condition. In our study, we highlighted the role of NGAL in bacterial induced sepsis, its mode of action, its inflammatory property, and its aid to cells in the prognosis of sepsis.

2. MATERIALS

The study system involved Healthy Human whole blood culture (HWBC), *E. coli* strain (ATCC25922): Culture medium Gibco TM RPMI 1600, LPS *Escherichia coli* O111:B4 (Sigma-Saint Louis, USA), TRI Reagent (Sigma-Aldrich India), SYBR Green (Applied Biosystems, USA), I Script cDNA synthesis kit, Bio-Rad Annexin V-FITC staining Thermo Fisher Scientific. Equipment and Software included: Applied Biosystem Real-Time PCR system, Flow cytometer GUAVA MILLIPORE Express Pro.

3. METHODOLOGY

3.1. Induction of *in vitro* Bacterial Infection

To determine in vitro inflammatory response blood was obtained from healthy human volunteers with informed consent for *in vitro* stimulation with *E. coli* O111:B4 LPS. Within 1 h of collection, 2 ml of heparinized blood samples were diluted 2:3 with RPMI 1640 supplemented with glutamine. Diluted blood was seeded into a 6-well microtiter plate and stimulated with 10 ng/ml LPS and maintained for 4, 6, 12, 18, and 24 h, respectively, at 37°C and 5% CO_2.

3.2. Effect of Steroid Hormones on NGAL Expression

To understand the inflammatory property of NGAL, HWBC was treated with different steroid hormones, Estrogen (E), Progesterone (P), Testosterone (T), and Adrenaline (A) at a concentration of 25 nM with/without LPS (10 ng/ml), respectively. All the aliquots were incubated at 37℃ with 5% CO_2 for 12 h and subjected to RNA isolation and qRT PCR analysis.

3.3. Effect of Retinol (Palmitate) on Upregulation of NGAL

Retinol in the form of palmitate (5 ug/ml) along with LPS (10 ng/ml) was added to HWBC and incubated for 12 h at 37°C and 5% CO_2, subsequently centrifuged at 1500×g for 3 min. Further serum was collected for western blotting and the rest of HWBC was subjected to RNA isolation and qPCR was performed and experiments were set up in duplicates by maintaining required controls.

3.4. Effect of Organic Root Extract of *Desmotricum fimbriatum* on NGAL

The organic root extract of the herb *Desmotricum fimbriatum (*DF) was extracted by methanol after an antihaemolytic assay was performed

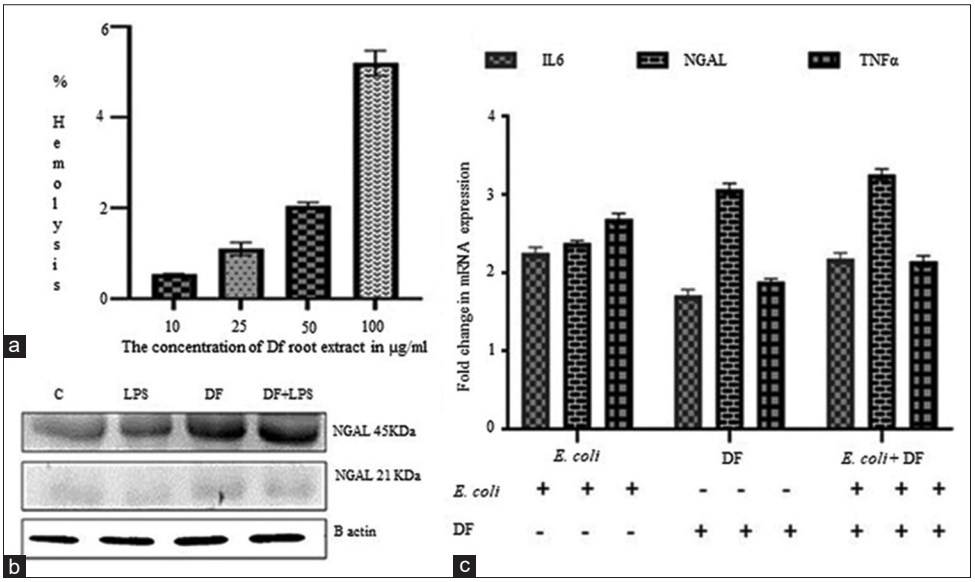

Figure 1: Effect of organic root extracts of Desmostricum fimbriatum DF on HWBC infected with E. coli. Anti-hemolytic assay on HWBC showed minimum hemolysis until 100 ∝*g*/ml (a), further 10 ∝*g*/ml of extract was used as the final concentration for treatment. Live *E. coli* cells at 5×10^2 CFU were inoculated into a 500 µl RPMI medium as a source of infection and a further 100 µl was used for inoculation. Thereafter, three groups were maintained in 2 *ml* of RPMI supplemented HWBC (1:4) with one set having only E Coli, the second set was with 10 µl/ml DF extract and the third set of cells was pretreated with DF extract for 2 and then infected with *E coli*. After 12 *h* of treatment, lysates were analyzed by western blotting (b) and q RT-PCR (c).

on HWBC [Figure 1a]. 10 µg/ml final concentration was chosen and the experiment was performed as follows. Two sets of HWBC diluted with RPMI (4:1) were pretreated for 2 h with 10 µg of DF extract an overnight culture of *E. coli* was serially diluted and a size of 5×10^2 CFU/500 µl was preferred for inoculation (100 µl) into above sets. In one set anti-NGAL antibody was added at 25 ng/ml. Positive control with only *E. coli* and negative control of HWBC was incubated along with test samples for 16 h at 37°C and 5% CO_2.

3.4.1. Apoptotic studies

Apoptotic studies for the DF extract-treated cells were processed for flow cytometric analysis. Where cells were spun down at 1500×g for 3 min, then washed with PBS once and 500 µl of binding buffer (0.03% $CaCO_3$ in PBS) was added and centrifuged at 250×g for 5 min. The pellet was added with 70 µl of binding buffer and 5 µl of Annexin V FITC stain and incubated for 15 min in dark. After incubation without washing the cells, 100 µl of binding buffer was added and cells were acquired for flow cytometry.

Table 1: Represents the set of primer sequences understudy.

S. No.	Genes	Primer sequence	Tm °C
1	NGAL	FP: 5'-CAAAGACCCGCAAAAGATGT-3' RP: 5'-GCAACCTGGAACAAAAGTCC-3'	51.78 50.73
2	IL6	FP: 5'-CCAGAGCTGTGCAGATGA-3' RP: 5'-CTGCAGCCACTGGTTCTGT-3'	57.1 57.9
3	TNFα	FP: 5'-CAGCCTCTTCTCCTTCCTGA-3' RP: 5'-AGATGATCTGACTGCCTGGG-3'	56.0 56.5
4	NF κ- β	FP: 5'-ATGGCAGACGATGATCCCTAC-3' RP:5'-CGGAATCGAAATCCCCTCTGTT-3'	54.4 54.8
5	TLR4	FP: 5'-GCCTCAGGGGATTAAAGCTC-3' RP: 5'-GTGAGACCAGAAAGCTGGGA-3'	56.8 55.5
6	IL10	FP: 5'-GCCACCCTGATGTCTCAGTT-3' RP: 5'-GTGGAGCAGGTGAAGAATGC-3'	56.4 57.1
7	STAT3	FP: 5'- CATCCTGAAGCTGACCCAGG-3' RP: 5'-TCCTCACATGGGGGAGGTAG-3'	55.9 55.9
8	PNOC	FP: 5'GAGACTGAGCAGCAGCAGGT LP: 5'TATGCTGGTGTGGCTGAGAA	56.2 57.5
9	GAPDH	FP: 5'-AAGGTGAAGGTCGGAGTCAA-3' RP: 5'-AATGAAGGGGTCATTGATGG-3'	55.3 57.3

3.4.2. Antibacterial studies

For Antibacterial studies, HWBC is treated with DF extract along with and without *E. coli* for 16 h and after which 50 µl of infected HWBC was taken and 150 µl of RPMI was added, the diluted HWBC were subjected to lysis in an ultrasonic bath for 3 min, and released bacteria 100 µl was spread plated on nutrient agar and incubated for 24 h and CFU/ml was determined. Simultaneously, the other part of HWBC lysate was studied for growth curve assay. The nutrient broth was inoculated with 5 0µl of treated HWBC and incubated at 37°C. Samples were drawn every 4 h up to 24 h and OD was measured at 600 nm and the graph was plotted against time in hours on the X-axis and OD at 600 nm on the Y-axis.

3.5. Isolation of RNA from Whole Blood

TRIZOL RNA extraction was performed on whole blood according to the manufacturer's protocol with a few minor modifications. The blood tube was inverted 3–5 times and to 1 mL of whole blood 1 mL of TRIzol Reagent was added and mixed thoroughly. The mixture was incubated at room temperature for 5 min before 0.2 ml of chloroform was added and the sample was inverted and incubated for 3 min at room temperature. This was followed by 15-min centrifugation at 12,000×*g* at 4°C. The upper aqueous phase containing the RNA was transferred to a new tube and 0.5 ml of Isopropanol was added and the sample was incubated for 10 min at room temperature, followed by centrifugation at 12,000×*g* at 4°C for another 10 min. The RNA pellet was washed with 0.5 ml of 75% ethanol and air-dried for 10 min before being resuspended in 30 µl of RNase-free water.

3.6. qRT-PCR Analysis

cDNA synthesis and qPCR were performed following the previously reported method, with slight modifications. cDNA was synthesized using the iScript cDNA synthesis kit (Biorad) using 1 µg of total RNA. The synthesized cDNA was mixed with SYBR Premix (Applied

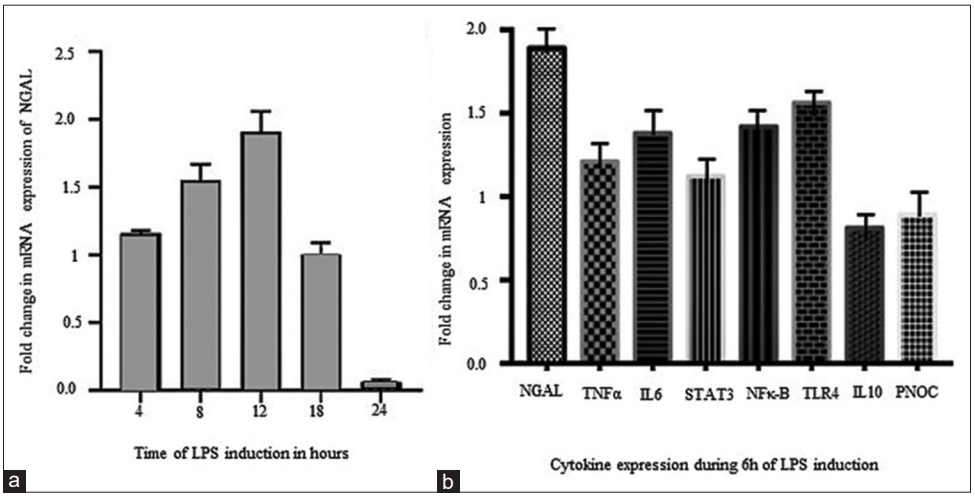

Figure 2: Effect of LPS on mRNA expression of NGAL and Acute-phase Response. *HWBC was induced with 10 ng of LPS and subjected to 4, 6, 12, 18, and 24 h of treatment. After subsequent treatments, RNA was isolated and subjected to qRT PCR. (a)* U*pregulation of NGAL mRNA expressed as early as within 4 h of LPS induction, and was consistent until 12 h, noticed until 18 h and then neutralized further. (b)* A *comparative study with known markers of sepsis IL6, TNFα, STAT3, NFKB, TLR4, IL10, and PNOC mRNA levels at 6 h. Data show upregulation of NGAL before all genes under study indicating an early requirement of NGAL necessary for mounting a* defense *against infection and also to exaggerate* acute *phase response.*

Table 2: Classification of inflammatory property of NGAL through the action of steroids.

S. No	Steroid understudy	Inflammatory Property Exhibited	Effect on NGAL mRNA expression	Classification of NGAL Acute Phase Response
1	Estrogen E	Anti-inflammatory	Suppressed by 0.52-fold	Pro-inflammatory
2	Progesterone P	Anti-inflammatory	Suppressed by 0.2-fold	Pro-inflammatory
3	Testosterone T	Anti-inflammatory	Suppressed by 0.35-fold	Pro-inflammatory
4	Adrenaline A	Anti-inflammatory	Suppressed by 0.58-fold	Pro-inflammatory

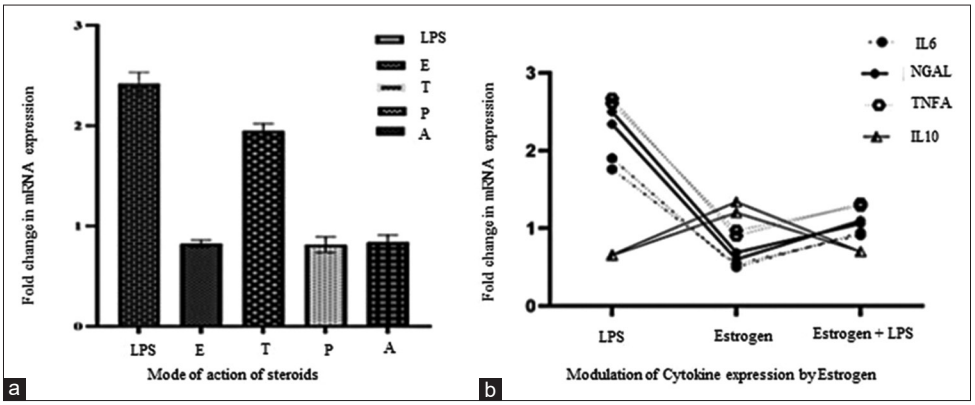

Figure 3: Effect of Steroid hormones on NGAL expression and modulation of Cytokines by Estrogen. HWBC were treated with 25nM of Estrogen (e), Testosterone (t), Progesterone (p), and Adrenaline (a) for 12 h after which RNA was isolated and Real-Time PCR was performed. (a) Represents changes in Cytokine expression in response to the hormone treatment. In (b), HWBC were subjected to costimulation with 10ng of LPS and 25 nM of estrogen, 25 nM of estrogen alone, and 10 ng of LPS alone for 12 h.

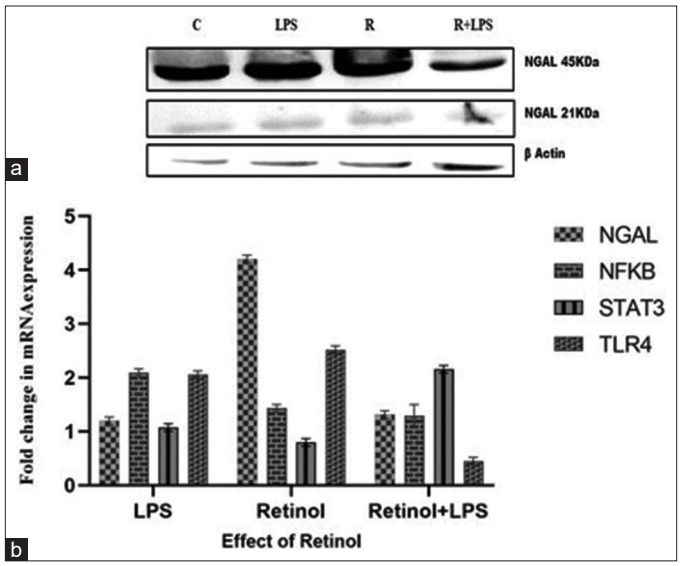

Figure 4: Effect of Retinol on NGAL in Correlation to NFκB and STAT3 under costimulation with LPS. HWBC were treated with 10 ng of LPS and/or retinol for 12 h and the lysates were probed for western blotting and qRT-PCR.

Biosystems) and specific primers. Amplification was performed for 35 cycles, each at 95°C for 15 s and 50°C for 1 min, 72°C for 1 min, and 95°C for 15 s using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The list of primers used is presented in Table 1. All measurements were performed in duplicate. NGAL upregulation was normalized against GAPDH. Further for comparative studies well established inflammation markers IL6, TNFα, IL6, STAT3, NFκB, TLR4, IL10, and PNOC were studied.

3.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, California USA software, values were subjected to multiple t-tests, one way ANOVA and two-way ANOVA analysis and represented as fold change in comparison to control. A significant difference of $P < 0.0001$ and $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. NGAL is an Acute Phase Response Protein

LPS treated HWBC cells subjected to q RT PCR showed elevated NGAL expression within 4 h of treatment [Figure 2a], establishing it as an acute phase polypeptide. Comparison with the inflammatory markers IL6, TNFα, STAT3, NFKB, TLR4, IL10, and PNOC revealed that elevation in NGAL expression could be detected much earlier at 4 h post-treatment, whereas the others could be detected at 6 h post-treatment [Figure 2b].

4.2. Classification of NGAL as a Pro-inflammatory Polypeptide through the Action of Steroids

To understand the mechanism of the early response of NGAL post LPS treatment, Estrogen (β Estradiol), Progesterone, Testosterone, and Adrenaline (25 nM/ml) treated HWBC were used. All the steroid hormones in the study were able to suppress the expression of NGAL [Table 2]. Estrogen in particular suppressed the expression of NGAL and TNFα to 1.1-fold and 1.23-fold, respectively [Figure 3a]. Whereas, IL10 an anti-inflammatory cytokine mRNA showed upregulation to 0.48-fold [Figure 3b].

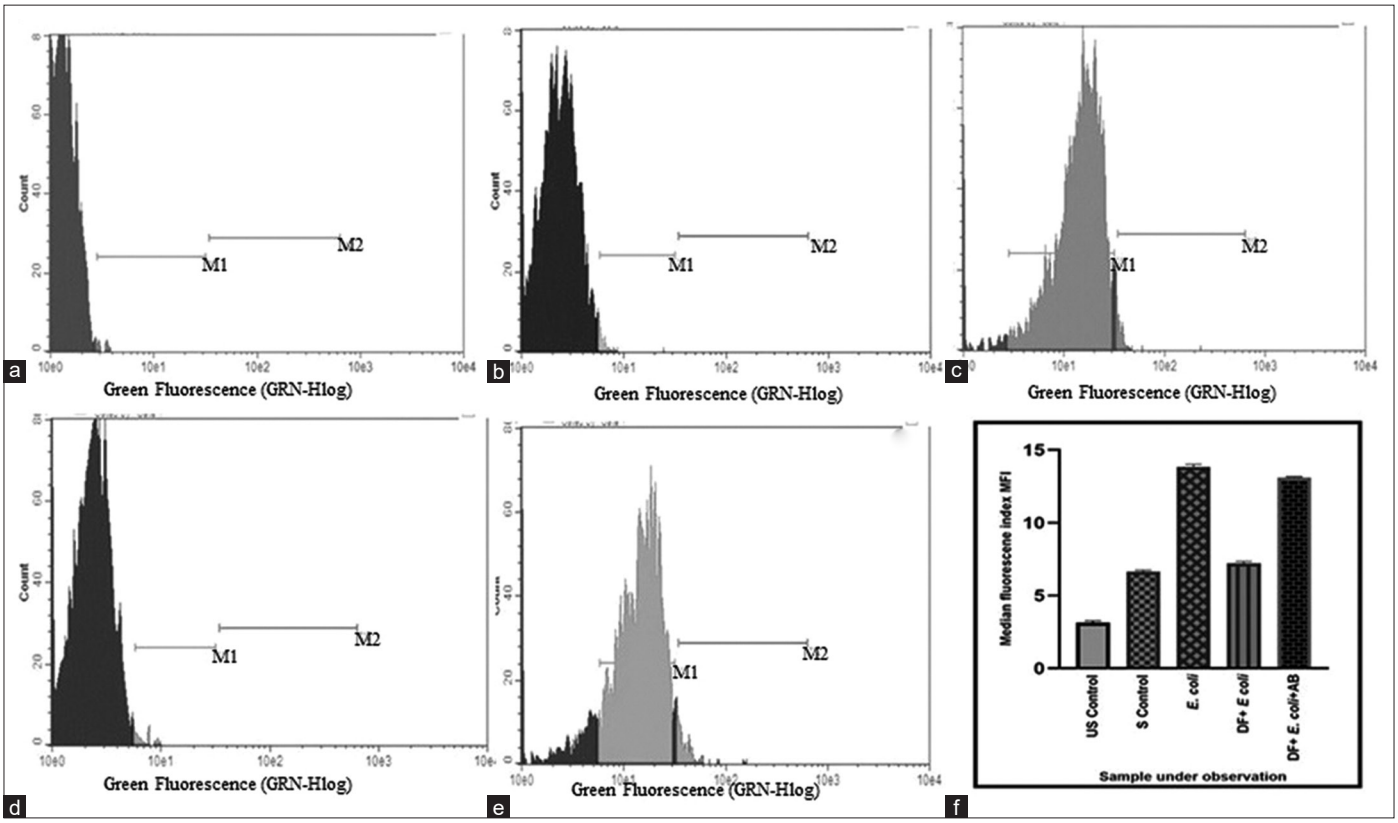

Figure 5: Apoptotic studies and Synergic effect of DF secreted NGAL protein on Live *E. coli* infected HWBC. Apoptotic studies were carried out by flow cytometric analysis of RPMI supplemented HWBC treated with live *E. coli* 100 CFU/ml. DF specificity for secreted NGAL protein was observed and included in the study. Five experimental groups were maintained as follows control, only *E. coli*, *E. coli* + DF (pretreated before 2 h), only DF, *E. coli* + DF (pretreated before 2 h) +NGAL Antibody (antagonist of secreted NGAL protein). After treatment for 12 h, cells were procured for Flow cytometric analysis. (a-e) Green fluorescence emitted by annexin FITC stain. Figure A unstained control cells (MFI 3.13), B - stained control cells (MFI 6.64), C-E coli infected cells (MFI 13.84), D-DF treated cells (MFI 7.225), E-E coli+ DF + NGAL antibody (MFI 13.11) and (f) shows cumulative MFI values which indicated DF shows a protective role which is reserved by treating with anti-NGAL antibody which shows induction of apoptosis.

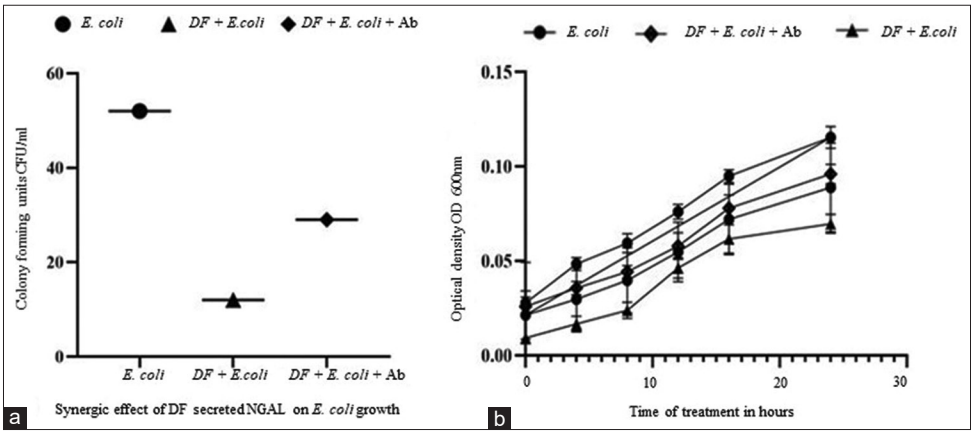

Figure 6: Anti-Bacterial effect of DF and NGAL protein on E coli multiplication. Growth curve assay was performed by maintaining four groups in RPMI supplemented HWBC (1:4), the first set was control, the second was *E. coli*, the third was DF (pretreated for 2 h) +*E coli*, fourth was DF (pretreated for 2 h) + *E. coli*+ anti-NGAL antibody. Samples were treated for 16 h after which *E. coli* cells were procured from infected HWBC by sonication. Lysates served as a source of E coli multiplication and were subjected to Agar plate assay for counting viable cells after treatment in CFU/ml. (a) CFU in all treatments (b) indicates the growth curve assay, where the lysates obtained were inoculated into 10 ml of nutrient broth. After inoculation samples were incubated at 37°C with adequate shaking for 24 h. Samples were drawn at 0, 4, 8, 12, 16, and 24 h, respectively, and optical densities were recorded at 600 nm.

4.3. Upregulation of NGAL through TLR4 dependent NFKB Transcribed Pathway

The role of retinol was investigated on NGAL expression post LPS treatment. Retinol treated cells showed upregulation of NGAL, NF-κB, TLR4, and suppression of STAT3 [Figure 4]. Although, retinol suppressed inflammatory cytokine and NGAL expression in the presence of LPS whereas STAT3 expression was observed to be reciprocal to the rest.

4.4. Therapeutics Intervention of *E. coli* Infection Through induced Upregulation of NGAL by DF Extract

DF extract was specifically able to induce NGAL protein production and was secreted into serum within 2 h post-treatment [Figure 1]. Further its role as an antiapoptotic and antibacterial factor was analyzed.

4.4.1. NGAL as an anti-apoptotic factor

The role of NGAL as an anti-apoptotic protein was investigated by flow cytometry. *E. coli* treated cells were observed to possess maximum Median Fluorescence Index (MFI 13.84), *E. coli* treated cells in the presence of DF extract showed protection from apoptosis with (MFI 7.22) which was reversed on co-incubation with anti-NGAL antibody (MFI 13.115) [Figure 5].

4.4.2. Antibacterial activity of DF secreted NGAL

DF treated HWBC subjected to growth analysis [Figure 6a] showed suppression of *E. coli* which was confirmed by a drastic reduction in counting colony formation units CFU/ml. HWBC treated with only *E. coli* showed 54 CFU/ml, whereas DF pre-treated cells, when infected with E. coli, showed 12 CFU/ml and finally, co-incubation of HWBC with *E. coli*, DF, and anti-NGAL antibody promoted multiplication of *E coli* (29 CFU/ml). Growth curve analyses indicated antibacterial activity of DF and NGAL since HWBC treated with *E. coli* and DF showed a drastic reduction in the growth of *E. coli* cells, which was reversed upon co-incubation with anti-NGAL antibody [Figure 6b]. These interesting findings indicate a specific protective effect of NGAL and DF in bacterial infection.

5. DISCUSSION

NGAL importance is implicated in several cancers [32], cardiovascular diseases [25], anemia [33], and also in kidney infections [22]. Its role in sepsis prognosis is unclear. Our study tries to ascertain its role and mode of action in LPS and live *E. coli* induced infection in human whole blood culture. Analysis of LPS induction on whole blood and regulation of NGAL revealed that LPS was able to modulate NGAL expression indicating that NGAL regulation followed TLR4 dependent upregulation. We could observe that NGAL can act as a biomarker even in Gram-negative bacterial infection since HWBC treated with LPS showed rapid elevation of NGAL expression within 4 h of treatment. This indicates that NGAL can be an excellent biomarker for infection like it is a known biomarker for kidney injury [13]. Early upregulation of NGAL in presence of both LPS and *E. coli* cells was observed as early as 4 h. A comparative study with known biomarkers such as IL6, TNFα [30] revealed that its upregulation is upstream to them and its early presence can serve as a source to build infection barriers, mobilize neutrophils, multiple cytokine signals [34] and also establish antimicrobial state [26] so that invasion of bacteria would be stationary [Figure 6].

To determine the survivability of sepsis patients, PCT (Procalcitonin) values are accounted for [35], similarly to understanding bacterial infection during early stages, NGAL can be employed as an early biomarker where medical intervention can be possible to save critical patients [36,37]. NGAL is a biomarker for Kidney injury where it is upregulated much earlier than creatinine [38]. NGAL is upregulated as early as within 2 h of injury, through which early medical attention can help prevent degeneration of the kidney and protect from organ dysfunction. A similar application can be sought in bacterial infection as well, as late detection of inflammation can lead to uncontrollable cytokine storms, organ dysfunction, and hence death. Hence, our study supports the evidence which proves that NGAL could act as a Biomarker even for bacterial infection and timely detection of it in serum and medical attention can save a person undergoing severity.

Further to understand its mode of action in serum after its elevation, the different external stimulus was selected where the steroid hormone effect was studied to delineate its inflammatory property. Estrogen, progesterone, testosterone, and also adrenaline treated whole blood HWBC induced with LPS were explored for their action on cytokines and prognosis of inflammation [39-42]. Among these, estrogen, progesterone, and adrenaline were observed to suppress NGAL expression. Estrogen in the presence of LPS was observed to have anti-inflammatory activity as it could lower inflammatory cytokine levels and also suppressed NGAL [Table 2]. A comparative study involving estrogen on mRNA of pro-inflammatory cytokines IL6, TNFα, anti-inflammatory cytokine IL10 and NGAL demonstrated NGAL as a pro-inflammatory peptide as it followed the pattern of suppression by estrogen to that of IL6 and TNFα [43,44]. Early expression of NGAL and its pro-inflammatory action could be important in building stronger defense mechanisms against invading bacteria by providing signals to the immune defense system to circulate and develop a strategy to fight against infection.

To understand the downstream regulation of NGAL, Retinol a JUN-KINASE inhibitor was used to stimulate HWBC induced with LPS [45]. NGAL has a site-specific for Retinol binding protein hence on exposure to retinol, upregulation of NGAL led to a 4.5-fold increase [Figure 5a]. Simultaneously transcription factors NFκB and STAT3 levels were also analyzed. Retinol showed an inhibitory effect on STAT3 mRNA expression when added; however, there was an upregulation of NGAL, NFKB, and also TLR4 m RNA expression indicating that NGAL would follow TLR4 dependent NFκB transcribed regulation but not STAT3 mediated regulation [Figure 4b].

After establishing the pro-inflammatory role of NGAL and its dependence on the TLR4 mediated NFκB dependent pathway, its role as an acute-phase protein was studied in response to an organic root extract of *Desmotricum fimbriatum* DF, a known herb with antioxidant properties [46]. We observed that extracts from *Desmotricum fimbriatum* could specifically enhance NGAL expression. DF extracts enhanced cell survival rate in *E. coli* infected HWBC, as observed by suppression of apoptosis Figure 5 This effect was reversed when cells were co-incubated with NGAL antibody [47].

Further, to study the antibacterial effect of secreted NGAL on the propagation of live *E. coli* inoculated HWBC was studied by subjecting the infected cells to sonication and sub-culturing the released bacteria by plating on the Nutrient Agar media. Measurement of Colony-forming unit CFU/ml related to the antibacterial effect of DF secreted NGAL since DF treated HWBC infected with *E. coli* showed the least number of CFU/ml of 12 as compared with only infected HWBC at 52 CFU/ml. This effect was

specifically reduced in treatments where an anti-NGAL antibody was added (29 CFU/ml) [Figure 6a]. Hence, NGAL suppression may be helping bacteria to prolong infection, inducing cells to undergo apoptosis. Whereas, DF mediated enhanced NGAL promotes an antibacterial effect, aided cell survival and inhibited bacterial propagation [26]. This demonstrates that pro-inflammatory markers like NGAL are important for cell survival and controlling infection and inflammation in the host.

6. CONCLUSION

Our study aimed at exploring the importance of Neutrophil Gelatinase Associated Lipocalin NGAL in Bacterial infection. Its importance is much stated during kidney injury but its prominence in bacterial infection is less underlined. In this study, we could establish NGAL as a prominent early biomarker of bacterial infection. An important observation here is that its upregulation is far earlier than prominent biomarkers such as IL6 and TNFα. Following its upregulation, NGAL works as a pro-inflammatory peptide trying to escalate the signals to mount a defense against ongoing infection. Alongside neutrophil recruitment, iron trafficking and signaling cytokine production would be also attributed to its function in aiding cell survival and preventing the progress of infection. To summarize the investigation, NGAL is an acute phase pro-inflammatory peptide following TLR4-NFκB transcribed regulation showing anti-apoptotic and antibacterial activity necessary for cell survival.

7. ACKNOWLEDGMENT

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8. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

9. FUNDING

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10. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

11. ETHICAL APPROVALS

Institutional Ethics committee of Prasad Reserach foundation has approved the study protocol. Approval number: ECR/998/Inst/TG/2017/RR-20/PRF15-2021, Approval Date: 15-November, 2021.

12. DATA AVAILABILITY

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

13. PUBLISHER'S NOTE

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