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Lymphoid and Myeloid Immune Cell Subsets Gene Markers and their Upregulation after Activation with Enterobacter Cloacae by using the Immune Cell Culture Technique

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Abstract: *Enterobacter cloacae* is a gram-negative, anaerobic bacterium that is capable of causing a variety of infections. Our study is focusing on the inflammatory role of Enterobacter *cloacae* across different immune cell cultures in vitro, including splenocytes and lymph nodes. The results of our study suggested a pro-inflammatory role of *Enterobacter cloacae,* inducing inflammatory gene markers of interest after cultivation with immune cells that were isolated from experimental animals(mice), mimicking the environmental human body conditions by using the immune cell culture technique. In our study, the fold change of proinflammatory gene markers of T lymphocytes, B lymphocytes, myeloid lineage and IL6 was investigated compared with the control group (inactivated immune cells group). Interestingly, the results of qPCR indicated a severe activation of all proinflammatory markers in gene expression, including CD3, CD19, GMCSF and IL6 in the activated group compared with the non-activated group. All in all, our results suggested the pro-inflammatory role of *Enterobacter cloacae* activated Lymphoid lineage and myeloid lineage, where these markers have been investigated in vitro by using immune cell culture technique.

Key Words: *Enterobacter Cloacae*, Inflammation, Lymphoid Lineage, Myeloid Lineage Gene Expression, Cell Cultur

INTRODUCTION

Enterobacter cloacae are anaerobic Gram-negative bacteria implicated in neonatal infections, which are linked to morbidity and multidrug-resistant infection [1]. They are recognized as pathogenic agents for respiratory and urinary tract infections. Recently, this species has emerged as one of the most prevalent nosocomial bacteria, particularly affecting patients with underlying conditions and those experiencing prolonged hospitalization, especially in the ICU and burn units. [2]

Across different types of Animal species, *E.Cloacae* has been recognized in a variety of animal species, including rabbits, mice and other animals where these bacteria playing an important role as either a commensal organism or an opportunistic pathogen.[3].

Enterobacter cloacae are anaerobic Gram-negative bacteria linked to neonatal infections, which are associated with significant morbidity and multi-drug resistant infections. Infections of the respiratory and urinary tracts are predominantly attributed to the pathogen Enterobacter cloacae [4]. Although they are regarded as commensals, members of the *E. cloacae* complex have the potential to function as opportunistic pathogens, resulting in significant infections in both healthcare settings and community environments, particularly in individuals with compromised immune systems. Enterobacteriaceae are characterized as a diverse group of Gram-negative rods that primarily inhabit the gastrointestinal tracts of animals and humans. These enteric bacteria, often referred to as coliforms, possess a complex antigenic structure and produce a range of toxins along with various virulence factors. *E. cloacae* and *E. aerogenes* have gained clinical significance as opportunistic bacteria, emerging as nosocomial pathogens particularly in intensive care patients, especially those on mechanical ventilation. Research indicates a significant relationship between the effect of

E. Cloacae and immune response in mice, highlighting the activation of both myeloid and lymphoid lineages in vitro [5].

METHODS

Enterobacter Cloacae Inoculation, Isolation and Identification

Two types of agars were used in this study including Blood agar (Oxoid,UK) and MacConkey agar (Oxoid,UK) which were both used to isolate *E. cloacae* which were then incubated aerobically at 37°C overnight, then non-lactose fermenting and oxidase-positive colonies were selected and sub cultured on *E. cloacae* agar (Himedia, India) and incubated using the same conditions. In order to identify the isolates, Analysis Profile Index (API) was used for identification of the isolates on the basis of their colony morphology, cultural characteristics, Gram stain, as well as multiple biochemical tests [6].

Experimental Animals-Mice

Mus musculus (Mouse) with in age 8-10 weeks which was obtained from the Animals House in the College of Veterinary Medicine at the University of Tikri and were these mice used as an experimental animal model .

Preparation of Cell Culture Media (Complete Medium)

Roswell Park Memorial Institute 1640 Medium(RPMI) was used with a volume of 90 ml which were added to 10 ml of Fetal Bovine Serum (FBS) then a volume of 5 ml of Pencilin/Streptomycin (PS) was added to the final volume which then well mixed before use [7].

Isolation of Splenocytes

A healthy mouse was kept under general anaesthesia, then euthanised, and the spleen was isolated and transferred into a specific bag containing 10 ml of complete media, which was in a container of ice. The bag which contains both the RPMI and spleen were then homogenized through stomacher blender (Biobase, China) which then filtrated through a specific filter (70 um) then the mix was treated with 600 ul of RBC lysis(Promega/ USA) for 45 -60 seconds to remove the erythrocytes, then the cells washed twice with Phosphate Buffer Saline (PBS) and a centrifuge was used with 1000 RPM for 10 minutes, Then the cells were seeded in a 96-well microtiter plate with 500,000 cells/well [8,9].

Isolation of the Mesenteric Lymph Node (MLN)

In order to isolate the mesenteric lymph node, the mouse was the target area of the isolation, and then isolated as described. In brief, a mesenteric lymph node was isolated from the abdominal cavity of the mouse, which was then transferred to specific bags that contain a volume of 10 ml of RPMI. Then a stomacher blender was used to smash the target organ to prepare single cells, which were then filtered by using a 70 µm filter and centrifuged at 1300 rpm for 10 minutes. The pellets were then resuspended in complete RPMI media. Then the cells were seeded in a 96-well microtiter plate with 500,000 cells/well for 24 hours in a CO2 incubator [8,9].

RNA Extraction and cDNA Synthesis

Both spleen and mesenteric lymph nodes were going through RNA extraction which the instructions were provided through Transzol up plus RNA Ki and then in order to assess the quality and concentration of the extracted RNA, a Nanodrop device (Thermo Fisher Scientific, United States) was used for this purpose , The extracted RNAs of both target organs then eluted in 20 ul of RNase-free water which then followed by synthesization through using 500 ng which was employed following the manufacturer's instructions. RNA was used as a template to synthesise the complementary strand using Transcript First-Strand cDNA Synthesis Super Mix Kit (Beijing, China) and following the steps recommended by the company.

Quantitative PCR (qPCR) Amplification

A trans start green qPCR super mix was used for this step. TransStart Green qPCR super mix, qPCR master mix containing the Trans Start Taq DNA Polymerase, SYBR Green, dNTPs, PCR enhancer and stabiliser, were used. The qTower3 qPCR machine from Analytika Jena (Germany) was used for the experiment, and all qPCR work was done in the central laboratory located at the College of Science/ University of Tikrit. The genes of interest studied are listed in Table 1. Gene expression was quantified, and specifically relative gene expression was quantified based on the housekeeping gene (GAPDH) and the genes studied from treated and control groups. The treatments were conducted in all immune cells from both spleen and mesenteric lymph nodes. The conditions for the cycling environment were as follows: 45 cycles, which consist of three steps at 98°C for 30 seconds,98°C for 10 seconds, and 60°C for 30 seconds.

cDNA concentrations were determined and normalised to the [10]. housekeeping gene in each sample using the ΔΔCT method [9,11].

Table 1: Primers used in the Current Study

Gene	Primer	Sequence 5'-3'	References
GAPDH	Forward Reverse	AGGTCGGTGTGAACGGATTTTG TGTAGACCAAGTAGTTGAGGTCA	Sultan *et al.* [9]
CD3	Forward Reverse	CGCAAAAGGACCTACGAGAC TGGGGGAAAACTCATCAAAG	Han *et al.* [21]
IL 6	Forward Reverse	GAACAACGATGATGCACTTGC TCCAGGTAGCTATGGTACTCC	Seetaraman Amritha *et al.* [10]
CD 19	Forward Reverse	ATTCGAGTGACAAGCCTGTAGCCCAC CTGGGAGTAGACAAGGTACAACCCA	Zhang *et al.* [23]

RESULTS

CD3 Marker

Our study indicated an activation in the proliferation of the immune cells which led to an upregulation in the gene expression of the proinflammatory gene marker Specifically Cluster of Differentiation 3 (CD3) which is inflammatory T cells Exclusive marker in both activated splenocytes and activated lymphocytes which were with *Enterobacter cloacae* in compared with the control inactivated group (immune cells only with RPMI media) as listed below in Figure 1 and 2.

IL-6 Marker

Another marker is IL-6, which is the characteristic Interleukin responsible of mediating signaling of inflammatory cells. This marker has been activated by *Enterobacter cloacae* in both spleen and lymph node where the results have shown that splenocytes were activated in spleen and lymph node compared with control unactuated group which is 1-fold in both of them as that show in Figure 3 and 4.

CD19 Marker

CD19 which is a cluster of Differentiation 19 which is a marker of B inflammatory cells which is mediated the humoral immunity exclusively. This molecule has been one of the markers of interest in this study that shown a huge response after activated with *E. cloacae,* where the splenocytes activated in spleen and lymph node compared with 1 fold only in the inactivated immune cells (control group) (Figure 5-6).

GMCSF Marker

GMCSF α is one of the important proinflammatory cytokines that mediates the inflammatory response for the myeloid lineage, including Macrophages and neutrophils, during infection, which is increased in the splenocytes and lymph node compared with 1 fold in the control inactivated group (Figure 7-8).

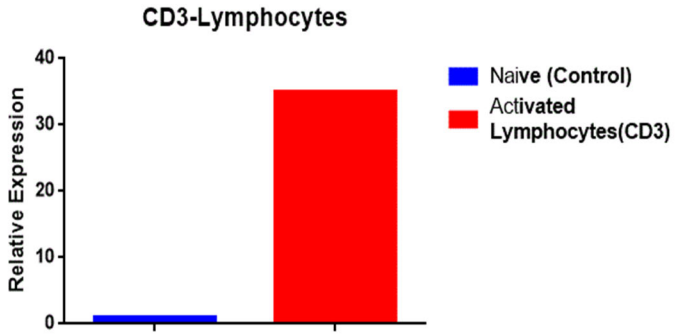

Figure 1: CD3-Lymphocytes before and after Activated with *E. Cloacae*

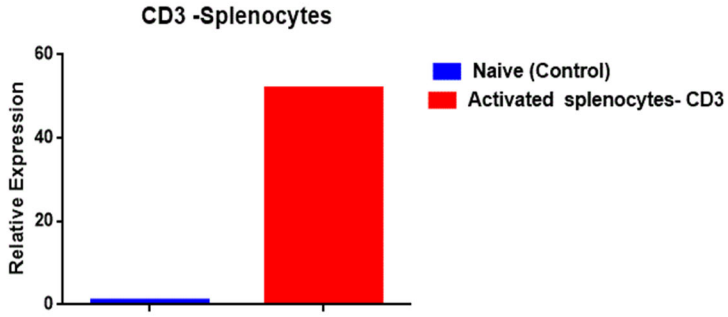

Figure 2: CD3-Splenocytes before and after Activated with *E. Cloacae*

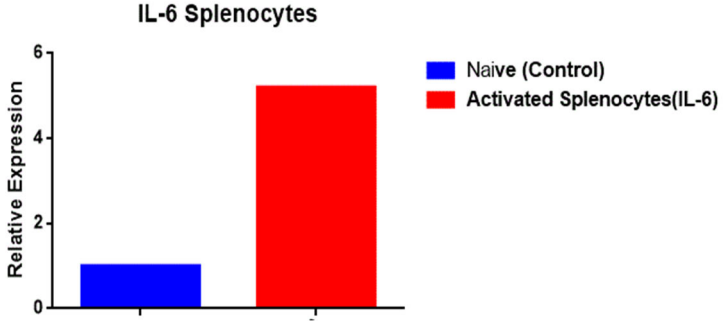

Figure 3: IL-6-Splenocytes before and after Activated with E. Cloacae

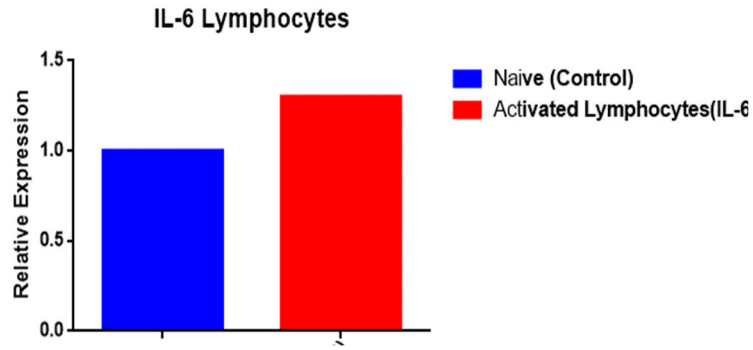

Figure 4: IL-6-Splenocytes before and after Activated with E. Cloacae

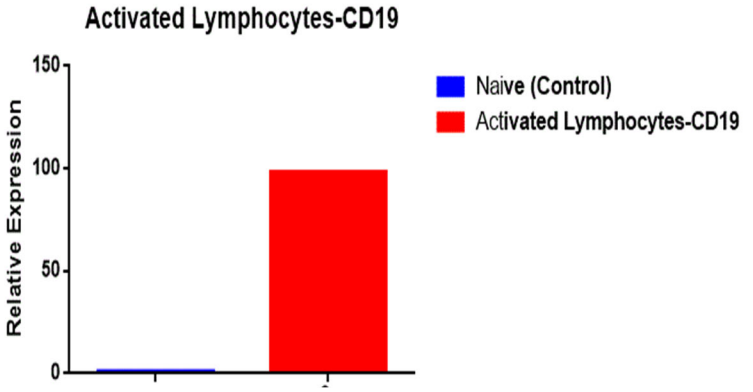

Figure 5: C19 Lymphocytes before and after Activated with E. Cloacae

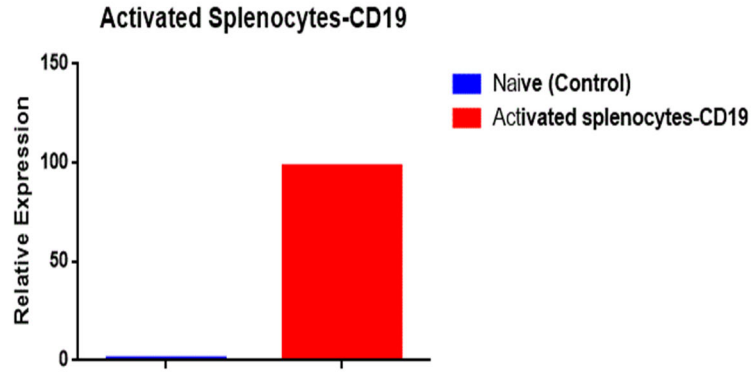

Figure 6: C19 Splenocytes before and after Activated with E. Cloacae

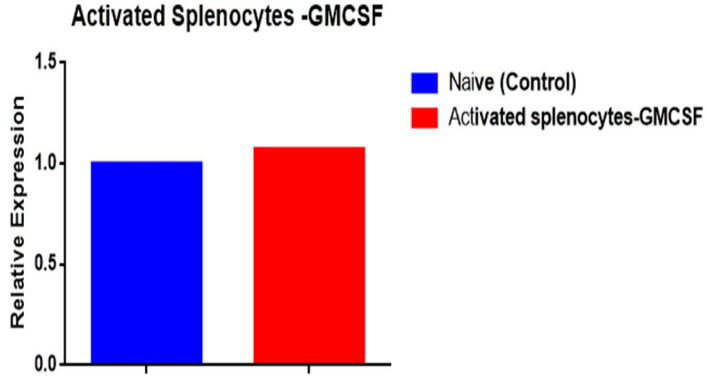

Figure 7: GMCSF Splenocytes before and after Activated with E. Cloacae

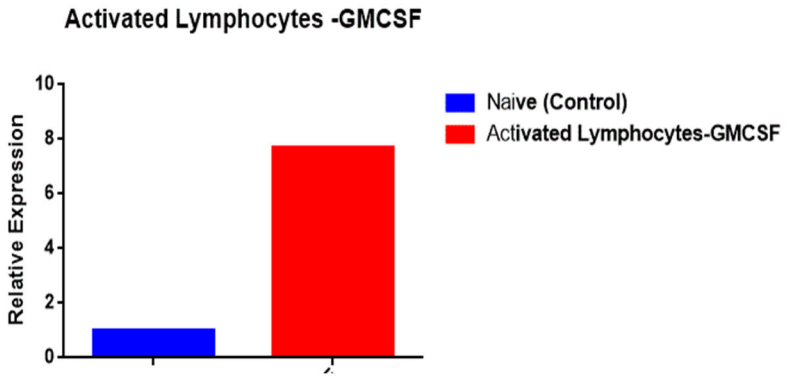

Figure 8: GMCSF Lymphocytes before and after Activated with E. Cloacae

DISCUSSION

Although *Enterobacter cloacae* complex is widely encountered in nature, they can act as a pathogenic bacterium as they mainly responsible for sepsis, urethritis, and respiratory tract infections[4]. The effect of *Enterobacter cloacae is* not only inducing infections but also may affect the transcription of the host and particularly their immune system [12]. while the biochemical and molecular studies on E. cloacae have shown genomic heterogeneity [13]

The studies have been noticed that Infections with E cloacae can trigger a strong immune response in myeloid lineage of immune cells where this lineage is considered as one of the both main lineages of immune cells in all creatures [14]. Neutrophils and macrophages are considering partner in crimes where phagocytic cells contributing to the primary line related to innate defense against bacterial pathogens through removing and destroying them at the epithelial barrier [15,16]. Innate lymphocytes cells and lymphocytes are both recognized as a component of Innate and adaptive immune system and they are considered as a source for Granulocyte-macrophage colony-stimulating factor (GM-CSF).[17].

Another marker which is IL6 Studies have shown heightened IL-6 levels in both systemic and localized infections caused by *E. cloacae* in animal models IL-6 is crucial for neutrophil recruitment to sites of inflammation [18] and these results support the results of our study where it has been shown that IL6 is crucial for activation of neutrophils as well as increasing inflammation markers where it has been increased in cultured immune cells of both spleen and mesenteric lymph nodes. Recent studies have shown that IL6, as a proinflammatory cytokine has been significantly increased both locally and systemically to gram negative bacteria locally and systemically as a response to infection with gram-negative bacteria [19].

CD19 a member of the immunoglobulin family where expressed exclusively on B lymphocytes, CD19 is a critical co-receptor for B cell antigen receptor (BCR) signal transduction [20]. Furthermore, alongside innate immune activation, the adaptive immune response involves the generation of immunoglobulins as mature B cells particularly plasma cells where they secreted antibodies against bacterial antigens [21] the results of these studies where g*et al*ong with the results of our study where it has been shown that CD19 gene expression where significantly upregulated in both splenocytes and lymphoid immune cells of the groups that activated with *E.Cloacae* compared with naïve group (unstimulated immune splenocytes and lymphocytes).

Monitoring levels of IL-6 and immunoglobulins which represent by CD19 can offer valuable insights into both the severity and progression of infections as well as the effectiveness of the host's immune response. These biomarkers also play a crucial role in assessing vaccine candidates or immunotherapies aimed at targeting E. cloacae and similar pathogens.

CD3 molecule is involved in T cell receptor (TCR structural stability as well as T cell activation signaling. CD3 is consider an exclusive marker of cellular immunity, antigen recognition as well as T cell signaling where all these facilitated by the TCR-CD3 complex , so the Potential pathogenicity of CD3 subunits can clarify the pathogenesis of immune system diseases and can offer fresh approaches to the treatment of it [22]. The results of our study indicated a significant increase of the CD3 expression marker in E, cloacae of both immune cells splenocytes and lymphocytes compared with naïve unstimulated immune cells [23].

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