Evaluate the Role of A. Baumannii in Promoting Inflammation and Meningitis-Related Genes in Various Immune Cells

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ABSTRACT
The role of Acinetobacter baumannii in promoting inflammation in spleenocyte and lymph node cells has been studied by detecting the gene expression of four different genes that are related to meningitis, which was performed after treatment of laboratory animal cells with dead cells of A. baumannii, the results of gene expression by using Real Time- Quantitative PCR and graph prism software exhibited the presence of statistical differences between A. baumannii treated and non-treated laboratory animal cells. All genes showed an expression between one and a half (1.5) to more than 40 (>40) fold change. The NLRP has expression in the spleen and lymph nodes between the (2-10) folds, which increase after being activated with killed A. baumannii cells. NF Kappa B has expression in the spleen and lymph nodes between (1.5-6) fold increase after being activated with killed A. baumanniicells. GMCSF has expression in the spleen and lymph nodes between (30-40) fold increase after being activated with killed A. baumannii cells. IL-1B has expression in the spleen and lymph nodes between (4.5-40) fold increase after being activated with killed A. baumannii cells.

1. Introduction

Acinetobacter species, specifically *Acinetobacter baumannii*, is the primary pathogen listed as critically important for developing new antibiotics. It is considered a "red-alert" human pathogen [1]. The success of *A. baumannii* as a newly recognized disease-causing organism is believed to be attributed not to the development of conventional harmful substances, such as toxins, but rather to a strategy known as "persist and resist." These pathogens can effectively infect their hosts because they possess a combination of molecular characteristics that enhance their ability to survive in the environment, such as resistance to drying out, the ability to form biofilms, and the ability to move. Additionally, they have recently been found to have virulence factors that contribute to their ability to cause disease, including secretion systems, surface glycoconjugates, and systems for acquiring essential nutrients [2]. These bacteria are gram-negative, motile, obligate aerobic bacteria with a rod-shaped capsule; they are found in the surrounding environment, such as water and soil, and grow at different temperatures and pH [3]. *Acinetobacter baumannii* has been frequently identified as the pathogen responsible for nosocomial meningitis in certain neurosurgical units. This is particularly true in patients with neurosurgical procedures like ventriculostomy insertion, drainage of cerebrospinal fluid (CSF), placement of monitoring devices, and lumbar drainage [4]. According to earlier research, 4.5% of meningitis in post-surgical patients and 11% in patients with intraventricular catheters were linked to *A. baumannii*. The limited penetration of antibiotics into the CSF makes treating meningitis caused by *A. baumannii*, which is resistant to drugs, a severe therapeutic issue [5]. Unfortunately, *Acinetobacter* meningitis is becoming an increasingly common clinical entity [6]. Cell culture techniques are methods that researchers use to study the growth and function of cells in a controlled environment. It's useful for studying gene expression, cell signaling, cell differentiation, and more [7]. Gene expression is how a gene's genetic information, or nucleotide sequence, can control a cell's structure and protein synthesis [8]. Therefore, by examining gene expression levels and the biological pathways linked to disease, it is possible to investigate the disparity between normal and infected cell pathways to ascertain the genetic source of the defective route [9].

2. Methodology

Preparation of dead bacterial cells

Growing *A. baumannii* colonies were collected from a specialized culture medium using a disposable loop. To kill the bacteria, the bacteria were mixed with 2 mg/ml of gentamicin and then

incubated at 37°C for three hours. The bacteria underwent centrifugation at 12,000 rpm for 5 minutes, then collected and stored at 4°C and suspended in complete media. As needed, the bacterial suspension was suitably diluted.

Mice

Mice (Mus musculus) (8-10) weeks of age, obtained from the Laboratory Animals House in the College of Veterinary Medicine at the University of Mosul, Iraq, were used for the study.

Ethical approval

Ethical approval for using animals in this research was given by the Tikrit University Health Research Ethics Committee, and all procedures were in strict compliance with set ethical standards. Thirty-six male mice weighing 85 and 145g were purchased From the animal house at Mosul University, and used for this experiment. Animals were housed in clean, well-ventilated cages for standard 12-h light and dark cycles at room temperature in the animal house in Mosul University, Iraq. Animals were fed standard rat chow and clean water ad libitum and allowed to acclimatize for 10 days before the start of the experiment.

Preparation of Cell Culture Media (Complete Medium)

9 ml of RPMI, 1 ml of fetal bovine serum (FBS), and 0.5 ml of penicillin-streptomycin solution were added to create the full medium. Before utilizing, the mixture was mixed well.

Preparation of immune cells isolation

The immune cells were collected from different organs, including the spleen and lymph nodes, which a specialized veterinarian obtained through dissection.

- Isolation of splenocytes: After aseptic mouse euthanasia, fresh splenocytes were obtained. In brief, erythrocytes were removed using RBC lysis solution, and splenocytes were then cultivated in complete media. The tissue was broken up into single-cell suspensions using a Stomacher blender. The cells were washed twice in a medium at 3000 rpm for 5 minutes. Next, 500,000 cells/well of a 96-well plate were seeded with the cells [10, 11].
- Mesenteric Lymph Node Isolation: Euthanized mice were used to isolate lymph nodes, as described by [12]. In brief, tissue was mashed via a Stomacher blender to create single cells in a complete medium. The cells underwent a 10-minute centrifugation at 1300 rpm after being filtered using a 70 µm filter. After resuspension of the pellet in a full medium, an automated cell counter was used to count the cells.

Specimen Preparation Procedure

After the spleen and lymph nodes were homogenized separately, the results were placed in different tubes, and 5 ml of the preservation medium was added. After that, the tubes were centrifuged for 7 minutes at 1300 rpm. After discarding the supernatant, the pellet cells were washed using 600 µL of RBC lysis solution. PBS was added to adjust the volume to 10 ml, and then the mixture was filtered using a particular filter. After that, the filtrate was centrifuged for 10 minutes at 1300 rpm. Following centrifugation, 1 ml of complete media was added to the sediment, and the supernatant was discarded. Then, 200 µl of the mixture was put into each well, and two wells were combined with 12.5 µl of the killed bacteria. The plate was then placed in an incubator for 48 hrs. After incubation, the cells were transferred from wells to separate Eppendorf tubes, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was removed, and 700 µl of Triazole was added to the sediment, which was then stored in a deep freeze for RT- qPCR [10].

Molecular Detection

Using cDNA generated from the total RNA of each sample, Real-Time Quantitative PCR (qPCR) was performed to analyze the mRNA expression of related genes for treated and non-treated

specimens. Then, the transgenebiotech company's Triazole Up Plus RNA Kit was used to assess the expression of the gene. The primers indicated in Table (3–12) were used for qPCR experiments.

RNA Extraction and cDNA Synthesis

The TriReagent (Transzol up plus RNA Kit) was used as directed by the manufacturer to extract total RNA from frozen samples. 20 µL of RNase-free water was used for the RNA elution, with the amount modified based on the residue's size. Thermo Fisher Scientific, in the United States, provided NanoDrop to assess the quality and concentration of the isolated RNA. The complementary strand (cDNA) was then synthesized using 500 ng of the extracted RNA using the TransScript First-Strand cDNA Synthesis SuperMix Kit (Beijing, China) according to the manufacturer's instructions.

Real-Time Quantitative PCR

TransStart Green qPCR SuperMix, a ready-to-use qPCR master mix containing TransStart Taq DNA Polymerase SYBR Green I, dNTPs, PCR enhancer, and stabilizer, was used for the quantitative real-time PCR. Relative gene expression was quantified based on the expression of housekeeping genes and the genes studied from the treatment and control groups. The Light Cycler 480 was used according to the manufacturer's instructions for the PCR procedure. The following were the cycling conditions: 45 cycles with three steps lasting 30 seconds at 98°C, 10 seconds at 98°C, and 30 seconds at 60°C. Three duplicates of the detected mRNA concentrations were determined, and each sample's mRNA concentrations were normalized to the housekeeping gene. The ΔCt technique was used to compare the different specimens.)[13, 14].

Table 1: Primer sequences used for RT-qPCR

Gene	Primer	Sequence
GAPDH Housekeeping	F R	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA
NLRP3	F R	ATTACCCGCCCGAGAAAGG CATGAGTGTGGCTAGATCCAAG
IL-1B	F R	GAAATGCCACCTTTTGACAGTG TGGATGCTCTCATCAGGACAG
NF-KB	F R	CGCAAAAGGACCTACGAGAC TGGGGGAAAACTCATCAAAG
GMCSF	F R	ACCACCTATGCGGATTTCAT TCATTACGCAGGCACAAAAG

3. Results and Discussion

The results of gene expression related to immune-meningitis markers using RT-PCR, show that there are significant differences between cell culture samples not treated with *A. baumannii* killed cells and cell culture activated with *A. baumannii*, the analysis was done using (Graph prism software). For spleen tissue, As shown in below figures, the results showed that all four genes being study have expression that increased after being activated with killed *A. baumannii* cells. The NLRP have expression in spleen more than two-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (1).

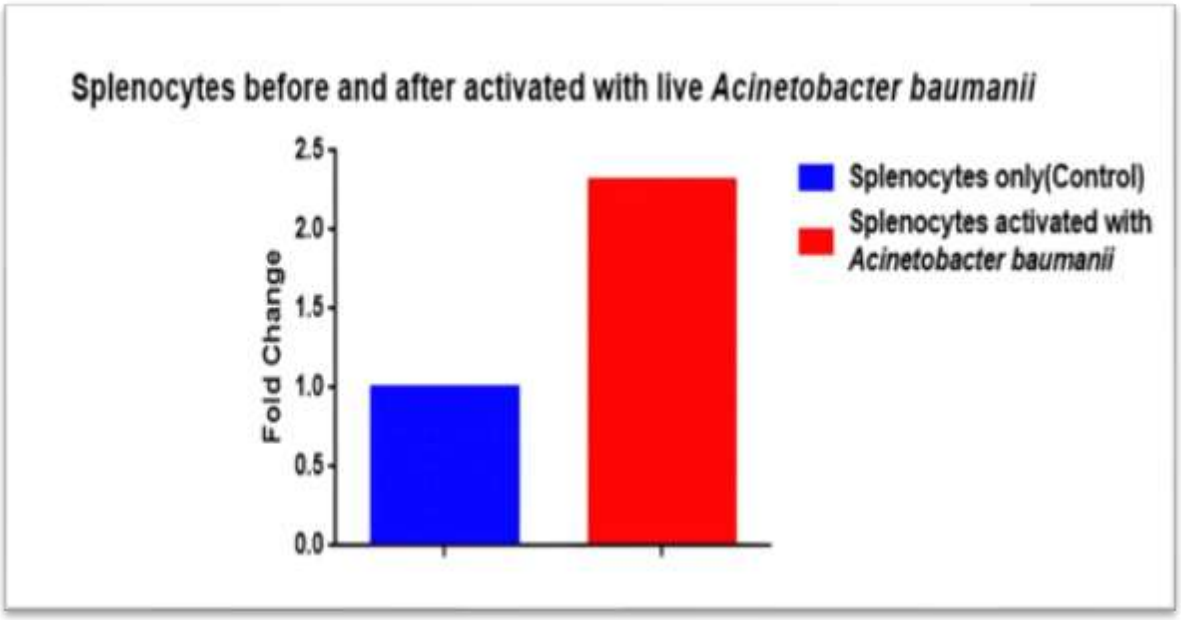

Figure 1: *NLRP* gene expression in spleen.

The NF Kappa B have expression in spleen more than six-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (2).

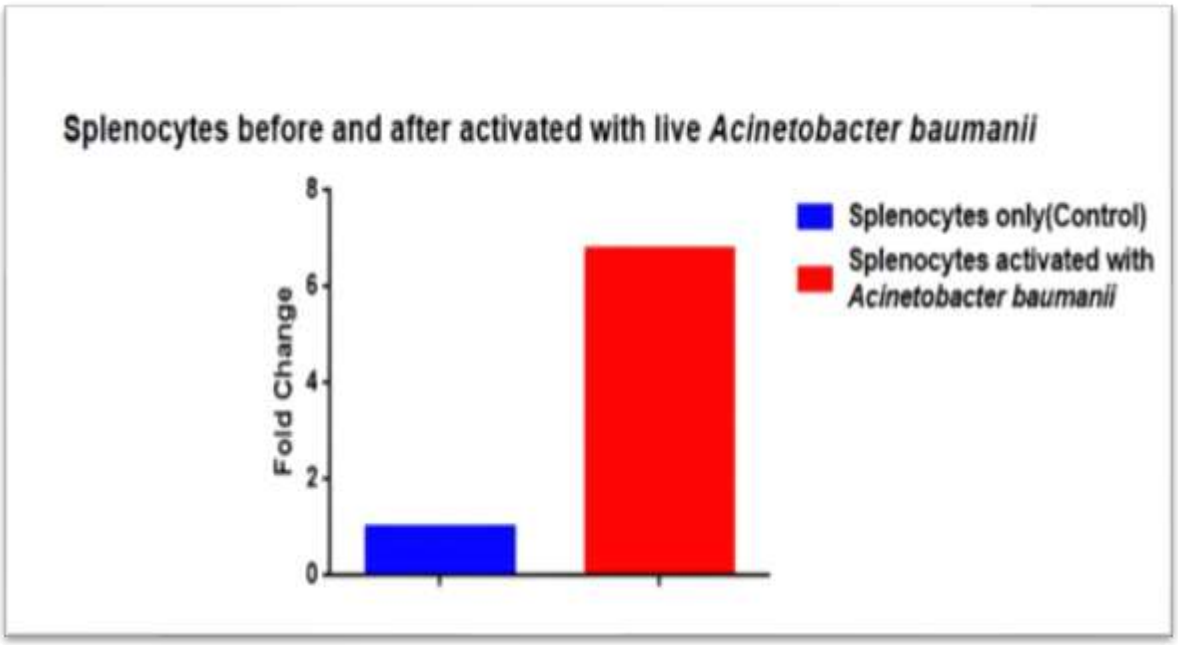

Figure 2: *NF Kappa B* gene expression in spleen

The GMCSF have expression in spleen more than 30-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (3).

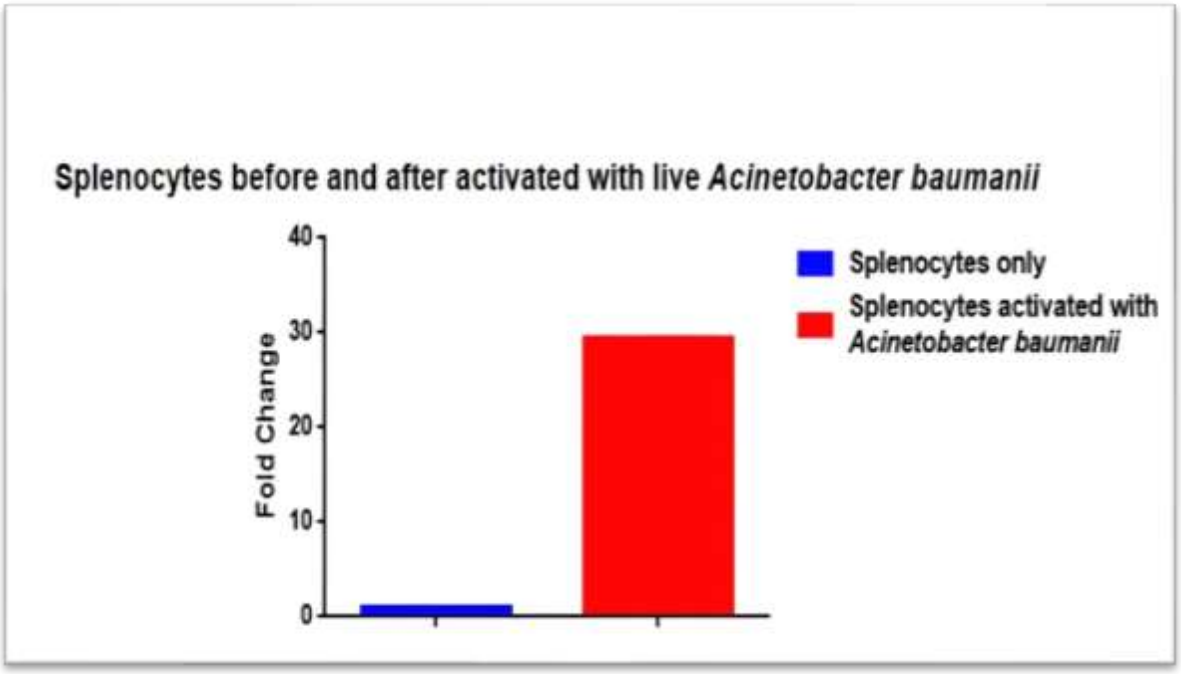

Figure 3: *GMCSF* gene expression in spleen

The *IL-1B* have expression in spleen more than four-fold increase after being activated with killed *A. baumannii* cells as seen in figure (4).

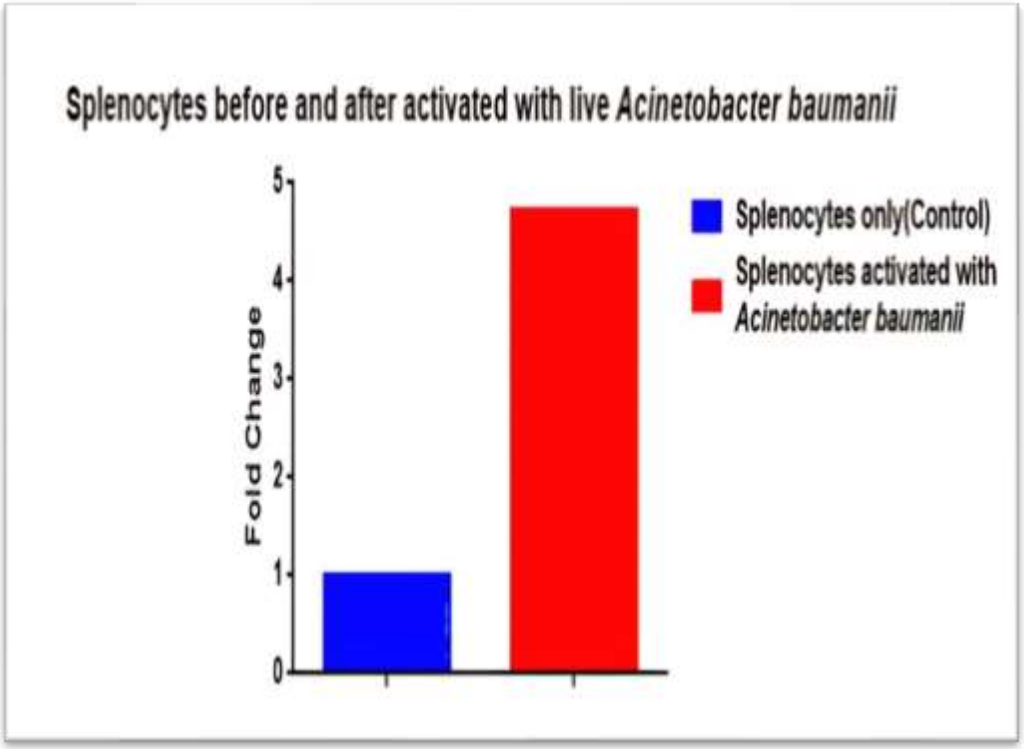

Figure 4: *IL-1B* gene expression in spleen

For lymph nodes, as shown in below figures, the results showed that three of the genes being study have expression that increased after being activated with killed *A. baumannii* cells, except one gene that has not changed. The *NLRP* have expression in lymph node more than 10-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (5).

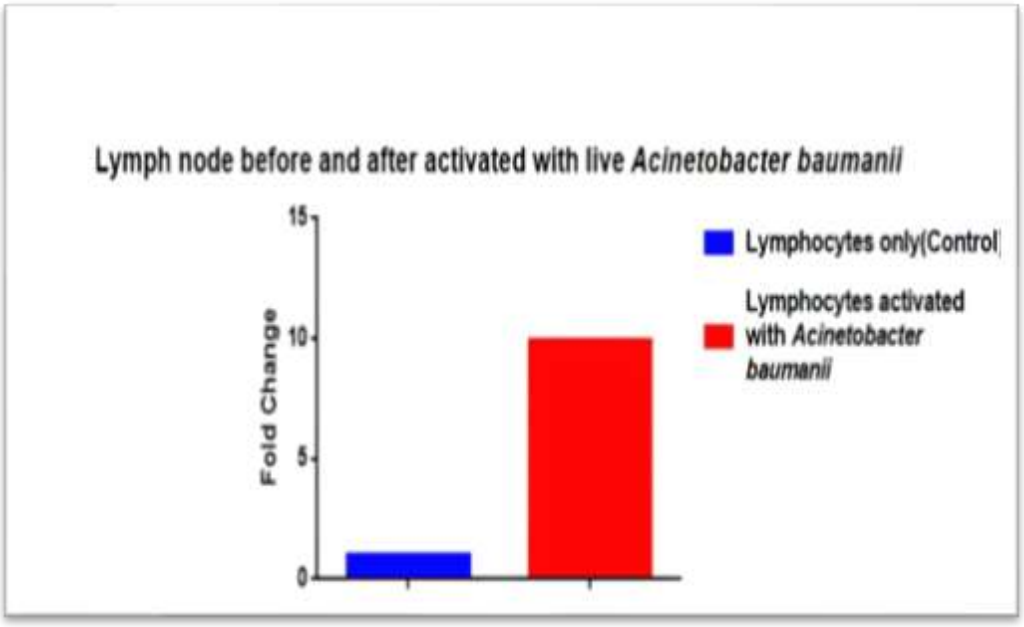

Figure 5: *NLRP* gene expression in lymph node

The *NF Kappa B* expression in lymph node changed very small percentage after being activated with killed *A. baumannii* cells as seen in Figure (6).

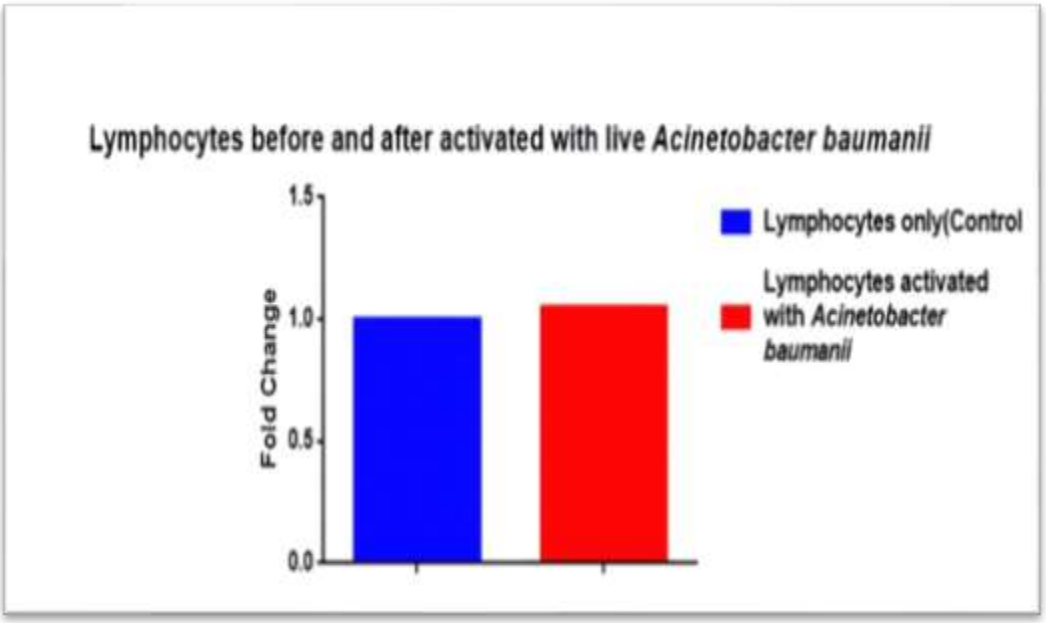

Figure 6: *NF Kappa B* gene expression in lymph node

The GMCSF have expression in lymph node about 40-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (7).

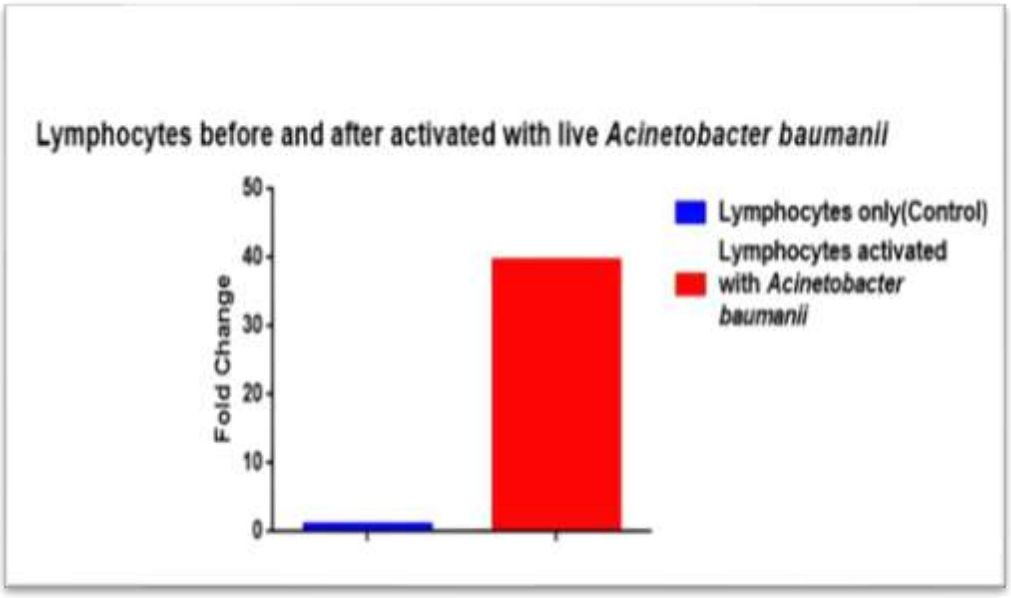

Figure 7: *GMCSF* gene expression in lymph node

The *IL-1B* have expression in lymph node about 40-fold increase after being activated with killed *A. baumannii* cells as seen in Figure (8).

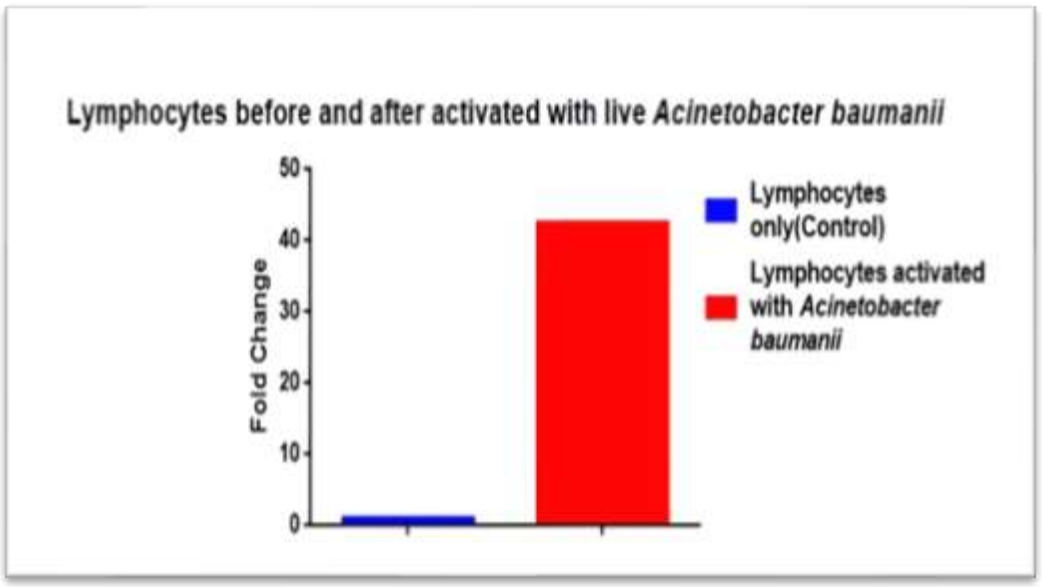

Figure 8: *IL-1B* gene expression in lymph node.

The inflammatory host response, triggered by bacteria in bacterial meningitis, is essential to its pathogenesis. Cytokines, including interleukin-1 beta, are the most significant components of this response. Production of cytokines in the CNS triggers a cascade of inflammatory mediators [15] Although the latest studies conducted by [16] indicate that the immune mechanisms resulting from the *A. baumannii* infection are elusive, our study aims to help bring about some development in this regard. According to a study conducted by [17], reactive oxygen species (ROS) generated from mitochondria are used by the *A. baumannii* pathogen pattern recognition receptor Omp34 to activate the NLRP3 inflammasome. Another study conducted by [18]shows that in mice with pneumococcal meningitis, the absence of the inflammasome components NLRP3, which are crucial for caspase-1 activation, reduces brain inflammation and decreases scores of clinical and histological disease severity.

The latest study [19] indicated higher levels of NLRP3 in the CSF in adults with bacterial meningitis. The findings of our investigation are corroborated by a study by Mitroulis *et al*.,[20], which stated that the function of NLRP3 in inflammatory conditions has already been established and is linked to an increase in gene expression. A study Mendiola *et al*.,[21] mentioned that in several CNS diseases, the proinflammatory cytokine interleukin-1 beta (IL-1β) has been linked to persisting immune responses and complicating the disease. Mook-Kanamori *et al*., [22] mentioned that during experimental pneumococcal meningitis, there is an up-regulation of Casp-1 mRNA and protein expression in the brain. This up-regulated activation is linked to elevated levels of IL-1β, and during pneumococcal meningitis, the Casp-1–IL-1β signaling pathways are essential for generating and amplifying the host inflammatory response.

McCracken *et al*., [23] measured IL-1b level in the CSF of a patient group made up only of newborns. Those authors found measurable amounts of IL-1b in the CSF in 95% of the cases at the time of diagnosis in 42 newborns with enterobacteria-caused meningitis, with quantities ranging from 20 pg/ml to 2616 pg/ml. In a very recent study conducted by Zhong *et al*.,[24] performed on children with meningitis to determine the influence of inflammatory factors on the effectiveness of adjuvant dexamethasone therapy in cases of refractory purulent meningitis, it was discovered that there were noticeably elevated IL-1 levels. Due to the increase in IL-1B levels between 4.5 - 40 folds in our study that examines changes in the gene expression that occurred in splenocytes and lymph nodes immune cells treated with *A. baumannii*, our study is consistent with previous studies. Various genes encoding proteins essential to immune response, inflammation, cell division, cell survival, and apoptosis are expressed by NF-KB. Therefore, the NF-KB signaling elevated under several pathological conditions [25, 26].

In meningitis, A common presentation of bacterial meningitis is the triad hallmark feature (THF): pathogen penetration, leukocyte transmigration at the blood-brain barrier (BBB), and nuclear factor-kappaB (NF-κB) activation in conjunction with type 1 interferon (IFN) signaling, resulting in high levels of cytokines and inflammatory cytokines in the blood and cerebrospinal fluid (CSF). Elevated NF-κB activation in CSF cells may indicate severe CSF inflammation [27]. According to the previously mentioned studies that reported NF-KB levels. This is consistent with the results we obtained in our research that examine changes in the gene expression that occurred in splenocyte immune cells treated with *A. baumannii*, which showed that NF-KB increased more than 6 folds compared to the control group. However, in lymph nodes' immune cells, the NF-KB changed in a very small percentage, almost non-existent, after being activated with killed *A. baumannii* cells for an unknown reason. This is why we recommend that there be studies on why it does not increase after infection with bacteria in lymph nodes' immune cells. For GM-CSF, a study by Amorim *et al*. (2022)[28] mentioned that during neuroinflammation, GM-CSF was the main target for destroying monocyte progenies. In multiple sclerosis (MS), GM-CSF is essential for the onset of the disease and the level elevated in the cerebrospinal fluid (CSF) of MS patients [29]. Wilejto *et al*.,[30] mentioned that there is evidence that GM-CSF plays a part in histiocytic diseases like Erdheim-Chester disease by stimulating the growth of myeloid cells. A recent study conducted by [31]on a 44-year-old male with cryptococcal meningitis, found that the GM-CSF level was 70-fold higher than normal. This study is consistent with the results we obtained that examine changes in the gene expression, which occurred in splenocytes and lymph nodes immune cells treated with *A. baumannii*, showed that GM-CSF increased between 30-40 folds compared to the control group.

4. Conclusion

This investigation determined that the pathogenesis of bacterial meningitis is contingent upon the activation of the inflammatory host response by bacteria. The most significant components of this response are cytokines, such as interleukin-1 beta. The expression of NLRP, NF Kappa B, GMCSF, and IL-1B in the spleen was significantly increased in activated cells with killed *A. baumannii* cells compared to the control group. The increases were more than two-fold, six-fold, more than 30-fold,

and more than four-fold, respectively. In the lymph node, the expression of NLRP, GMCSF, and IL-1B has increased by over tenfold, fortyfold, and fortyfold, respectively, following the activation of *A. baumannii* cells that have been killed. After being activated with killed *A. baumannii* cells, the expression of NF Kappa B in the lymph node changed by a negligible percentage.

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