**INTRODUCTION**

Inflammation is the immune system’s response to a damage caused in cells or tissues by pathogens or by any biological, chemical, physical, or mechanical aggressor. Inflammation is a generic response and therefore is considered a mechanism of innate immunity, compared to adaptive immunity, which is specific for each pathogen. The pandemic coronavirus infectious disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), nowadays suppose a huge problem for whole world. The principal characteristic of this virus is that in some cases it can decrease IFN-γ expression in CD4+ T cells, cause the lymphopenia (in CD4+ and CD8+ T cells) but at the same time it increases drastically pro-inflammatory cytokines (“cytokine storm”). All these immunological dysregulation characteristics are typical in severe COVID-19. Interferons (IFNs) are a family of cytokines which, upon secretion, play a central role in mediating the innate and adaptive immune response to viral and bacterial challenges. There are now two IFN family members identified; Type I IFN, which include IFN-α and IFN-γ, and Type II IFN which is comprised of IFN-γ. Both of which have distinct physiological roles, bind to different receptors, and are structural diversity; however, both Type I and Type II IFNs activate the anti-viral response. Moreover, Type I IFNs are secreted following the activation of intracellular and extracellular anti-viral sensors, mainly by macrophages and dendritic cells whereas Type II IFN is primarily secreted by T lymphocytes and natural killer (NK) cells in response to cytokine activation, specifically IL-12 or IL-18, with stimulation from both IFN subtypes leading to downstream cytosolic signaling and subsequent upregulation of gene expression. The exaggerated level of cytokines such as IL-6, IL-1, TNF-α among others might be responsible that symptoms like severe weakness/tiredness, myalgia, headache, breathing difficulties or lung damage appears. Interleukin 6 is a potent inflammatory cytokine that exhibits functional pleiotropy in numerous cell types, contributes to host defense against infections and tissue injuries. However, the excessive

**ABSTRACT**

The aim of the present study was to evaluate in vitro the anti-inflammatory effects of two products: a liposomal lactoferrin (LLF) solution versus a liposomal lactoferrin-zinc complex (LLF-Zinc) on CD163 and CD14 protein levels, and gene expression of Tumour Necrosis Factor alpha (TNFα), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6) and Interferon Gamma (IFNγ), after treatment in Peripheral Blood Mononuclear Cells (PBMCs) directly extracted from human volunteers.

Treatment with Liposomal lactoferrin and liposomal lactoferrin plus zinc (LLF-Zinc) in Peripheral Blood Mononuclear Cells (PBMC), activates a cytokine response mediated by IFNγ, that increases defense mechanisms against infection (induced by LPS). This mechanism seems to be different from that triggered by LPS, precisely because of the specific activation of IFNγ by the two products. Interferons (IFN) are a family of cytokines which, upon secretion, play a central role in mediating the innate and adaptive immune response to viral and bacterial challenges. LLF and in specially LLF-Zinc stimulates Interferon anti-viral responses enhancing our natural defenses against microbial insults.

**Keywords:** Liposomes, Lactoferrin, Zinc, Immunity, IFNγ.
synthesis of IL-6 causes acute severe systemic inflammation; also, is an early biomarker of lung injury and predictive factor of prolonged mechanical ventilation, organ dysfunctions, morbidity, and mortality in lung diseases.\(^{10}\) CD163 (Cluster of Differentiation 163) is the high-affinity scavenger receptor for the hemoglobin-haptoglobin complex and it is nearly exclusively expressed in monocytes and macrophage cells. During acute inflammation and when the macrophages are activated, sCD163 levels increase extremely due to metalloproteinase-mediated cleavage near the cell membrane. The function of sCD163 is not known very well, but it is used as a biomarker of macrophage activation in some inflammatory diseases like sepsis or liver disease.\(^{10}\) Also, it serves as a marker of macrophage activation after Ebola virus infection in humans.\(^{10}\) Some authors describe that CD163 acts as innate immune sensor for bacteria,\(^{11}\) and viral infection, for respiratory syndrome viruses.\(^{12}\) CD163 is upregulated by IL-6, IL-10, and glucocorticoids, while it is downregulated by proinflammatory cytokines like TNF-α, interferon-gamma (IFN-γ) and the chemokine CXCL4 (platelet factor 4).\(^{3}\) Some medication and bioactive compounds with anti-inflammatory effect were proposed for treating the symptoms and complication of Sars-CoV-2 virus. For example, Serrano et al. used liposomal lactoferrin and liposomal zinc in symptomatic persons positive in COVID-19. The results of this study suggest that oral treatment with Liposomal Lactoferrin (LLF) and LLF + Liposomal Zinc (LZ) could be helpful as a co-adjuvant treatment for fast recovery of patients with COVID-19 within the first 4-5 days.\(^{15}\) Lactoferrin is a glycoprotein naturally presented in milk with a multiple function like immunomodulating effect and when the macrophages are activated, it serves as innate immune sensor for bacteria,\(^{11}\) and viral infection, for respiratory syndrome viruses.\(^{12}\) Lactoferrin can block the binding of Sars-CoV-2 spike protein to HaCaT cell receptors in the in vitro study.\(^{16}\) In another in vitro study in African green monkey kidney epithelial cells (Vero E6) and in adenocarcinoma human alveolar basal epithelial cells (A549) infected with Sars-CoV-2 virus was confirmed the inhibitory effect of lactoferrin on the virus.\(^{16}\) Considering the potential effect of LLF for treating the symptoms in COVID patients due to exaggerated inflammation and immunological dysregulation caused by this virus, in this study we investigated the anti-inflammatory and immunomodulating potential of Liposomal Lactoferrin and Liposomal Zinc Sulfate through regulation of the LPS-induced expression of 4 genes (TNFα, IL-1β, IL-6 and IFNγ) and inhibition of 2 proteins (CD14 and CD163) directly involved in inflammatory pathways, after in vitro treatment on Peripheral Blood Mononuclear Cells through RTqPCR quantification.\(^{17}\)

**MATERIAL AND METHODS**

**Products tested**

The products that were tested: Liposomal Lactoferrin (LLF) and Liposomal Zinc Sulfate (LZS). The compounds were stored at 4°C in our facilities to avoid alteration and dilutions were prepared freshly each time.

**Platform**

Peripheral Blood Mononuclear Cells (PBMC) extracted and isolated from human volunteers.

**Analytical equipment**

Microscope, incubator, refrigerated centrifuge, statistical analysis software, micropipettes, pipettes, propipettes, rack, quantifier Nano-Drop spectrophotometer, laminar flow hood, analytical balance, microcentrifuge, Synergy H1 Hybrid Multi-mode plate reader, Quant studio 5 (Applied Biosystem) Quantitative real-time PCR, vortex, Bürker chamber, freezer -80°C, freezer -20°C, heating block, thermocycler and general and specific consumables for PBMC isolation (SepMat PBMC Isolation Tubes- SC Technologies).

**Reagents**

Distilled water (Braun), PBMC specific medium (RPMI) and supplements, Gentamicin (Sigma), Lymphoprep Medium for PBMC isolation (SC technologies), Phosphate buffered saline (Sigma), Trypsin/EDTA (Sigma), Trypan Blue Solution (Bio-Rad), DMSO (Sigma), MTT powder (Invitrogen), RNeasy extraction kit (Qiagen), DNase-I (Qiagen), PrimeScript RT reagent kit (Perfect Real Time-Takara Clontech), oligonucleotides for RT-PCR amplification of TNFa, IL-1β, IL-6 and ACT, SYBR ® qRT-PCR, Human CD163 ELISA Kit (M130) and Human CD14 ELISA Kit (Abcam).

**Procedure**

Peripheral Blood Mononuclear Cells (PBMC) extraction from human volunteers.

20 ml of human blood were extracted from a volunteer in EDTA-Tubes. Blood was centrifuged and serum was substituted by PBS. After that, the mixed sample was added drop by drop in SepMate tubes containing 3.5 ml of Lymphoprep Medium. Tubes were centrifuged again, and supernatants were isolated to a fresh standard tube. Cells were washed twice in RPMI medium and counted before seeding.

**Cell viability – MTT assay**

To determine live cell count, cell viability was checked by staining with Trypan-Blue Solution (the blue compound can only penetrate dead cells). Live cells were counted in a Bürker chamber under the microscope. For MTT assay, PBMC cells were cultured overnight at 10,000 cells/well density in a 96-well plate. 24 hours later, cells were treated with different concentrations of LLF or LLF + LZS (0.05%, 0.01%, 0.005%, 0.001%, 0.0005%, 0.00005% and 0.00001%) diluted in growth medium. After 24 hours of incubation, medium was removed, and wells were washed with PBS to eliminate any residual medium, and a solution of MTT 1:11 was added to each well. Plates were incubated in the refrigerated incubator at 37°C for 3 hours. MTT reactive was removed, and DMSO 100% was added to each well to solubilize formazan crystals prior to absorbance measurements at 550 nm and reference of 620 nm.

**Protein analysis – ELISA assay**

Peripheral Blood Mononuclear Cells were seeded in 48 plates (30,000 cells per well). 24 hours later, cells were treated with LLF or LLF + LZS at 0.01% or 0.05% concentrations. Medium for control replicates was replaced by fresh medium without treatment. After 30 minutes of incubation, LPS at 150 ng/ml concentrations was added to the culture medium for inflammatory response induction. After 24 hours of incubation, supernatant was collected from all samples and CD163 and CD14 protein levels were quantified following ELISA kit and manufacturer’s instructions. Results were obtained measuring absorbance at OD 450 nm. 5 technical replicates per condition were included in each of the assays.

**Gene expression analysis – RT-qPCR (24 hours)**

For the main gene expression assay, PBMC cells were cultured at a 30,000 cells/well density in a 48-well plate, in growth medium, 24 hours later the medium was removed and LLF or LLF + LZS at 0.01% and 0.05% concentrations were added to the cells. After 30 minutes of incubation, LPS at 150 ng/ml concentrations was added to the culture medium for inflammatory response induction. After 24 hours of incubation, cells were washed with PBS buffer and collected in...
lysis buffer to proceed with RNA extraction. Total RNA was extracted using RNeasy kit (Qiagen) and treated with DNAsel-1 to remove any contamination from genomic DNA. RNA quality and quantity were checked in a Nano-Drop spectrophotometer, and 1 mcg of total RNA was used to synthesize cDNA, using First-strand Synthesis kit (Takara-Clontech). The suitability of each primer pair used in this study for RT-qPCR, TNFα, IL-1β, IL-6, IFNγ and ACT was previously evaluated to determine melting curves, efficiency of amplification and specificity of the primers. Finally, quantitative PCR (qPCR) was performed in a real-time PCR machine (QuantStudio 5, Applied BioSystem). To perform raw data analysis, we used the Pfaffl method[18] to calculate the gene relative expression ratio to ACT (internal control- housekeeping gene). Mathematical model of relative expression ratio in real-time PCR. Statistical analysis to determine significant changes was performed using ordinary one-way ANOVA and unpaired Student’s t-test. For all data, a level of 5% or less (p < 0.05) was taken as statistically significant.

**Gene expression analysis – RT-qPCR (30 min and 2 hours)**

For the main gene expression assay, PBMC cells were cultured at a 30,000 cells/well density in a 48-well plate, in growth medium, 24 hours later the medium was removed and LLF or LLF + LZS at 0.0001% and 0.001% concentrations were added to the cells. After 30 minutes of incubation, LPS at 300 ng/ml concentrations was added to the culture medium for inflammatory response induction. After 30 minutes or 2 hours of incubation, cells were washed with PBS buffer and collected in lysis buffer to proceed with RNA extraction. Total RNA was extracted using RNeasy kit (Qiagen) and treated with DNAsel-1 to remove any contamination from genomic DNA. RNA quality and quantity were checked in a Nano-Drop spectrophotometer, and 1 mcg of total RNA was used to synthesize cDNA, using First-strand Synthesis kit (Takara-Clontech). The suitability of each primer pair used in this study for RT-qPCR, TNFα, IL-1β, IL-6, IFNγ and ACT was previously evaluated to determine melting curves, efficiency of amplification and specificity of the primers. Finally, quantitative PCR (qPCR) was performed in a real-time PCR machine (QuantStudio 5, Applied BioSystem).

All data were statistically analyzed using ordinary one-way ANOVA test and unpaired Student’s t-test. Statistical significance was set at p < 0.05, 95% of confidence. Data were represented in bar graphs as Mean ± Standard Error of the Media (SEM). Statistical significance is shown in bar graphs according to ordinary one-way ANOVA test.

**RESULTS.**

According to previous assays, 0.01% and 0.05% concentration were selected for further protein and gene expression studies.

**Protein levels**

Results indicated the treatment with bacterial LPS significantly increased CD163 and CD14 protein levels, compared to the untreated control, meaning the LPS is effectively inducing inflammation through these inflammatory pathways. When treatments with LLF at 0.05% or LLF + LZS at 0.01% and 0.05% concentrations were also applied, results indicated significant inhibition of CD163 protein levels by 43.6 ± 8.6%, 33.7 ± 9.0% and 56.2 ± 10.9%, respectively, compared to the Control + LPS. Results for CD14 showed the treatment with LLF + LZS at 0.05% concentration decreased protein levels by 20.6 ± 10.2%, even though results were not statistically significant (p > 0.05), compared to the untreated control; as shown in Figure 1.

**Gene expression levels (24 hours)**

**Gene expression analysis**

mRNA expression levels were determined for the products tested in Peripheral Blood Mononuclear Cells (PBMC), after treatment for 24 hours, in parallel to inflammatory response induction with LPS. TNFα, IL-1β, IL-6, IFNγ and ACT (internal control) were amplified using four technical replicates of cDNAs. Results indicated the treatment with LPS significantly induced the expression of genes IFNy, IL-1β, aD and IL-6, meaning the LPS is effectively inducing immunological response through these inflammatory pathways. When treatments were also applied in parallel, results indicated the treatments with LLF or LLF + LZS significantly increased the expression of most of the genes, for both tested concentrations; as shown in Figure 2.
Gene expression levels (30 min and 2 hours)

mRNA expression levels were determined for the products tested in Peripheral Blood Mononuclear Cells (PBMC), after treatment for 30 minutes and 2 hours, in parallel to inflammatory response induction with LPS. TNFα, IL-1β, IL-6, IFNγ and ACT (internal control) were amplified using four technical replicates of cDNAs.

Results indicated the treatment with LPS significantly induced the expression of genes TNFα, IL-1β and IL-6, meaning the LPS is effectively inducing immunological response through these inflammatory pathways. When treatments were also applied in parallel, results indicated the treatments with LLF or LLF + LZS for 2 hours significantly increased the expression of most of the genes, for both tested concentrations; as shown in Figure 3. However, when treatment was performed for 30 minutes, TNFα gene expression was significantly decreased by LLF and LLF + LZS treatments at 0.001% concentration; as shown in Figure 4.

DISCUSSION AND CONCLUSIONS

Inflammation is the immune system’s response to a damage caused in cells or tissues by bacterial pathogens or by any other biological, chemical, physical, or mechanical aggressor. Cytokines are a family of proteins and glycoproteins that regulate the inflammatory and immune response, most of them are produced by epidermal cells. These cytokines are soluble molecules acting as chemical mediators released during the process, which help to intensify and propagate the inflammatory response; frequently including TNF-α, CCL2/MCP-1, C-RP, IL-1, IL-6, INF-γ, DUOX1, CXCR4 (SDF1 receptor), histamine, IL-10, TGF-β, COX-2, PGE2, etc. Although it’s role in the inflammatory process is complex, these molecules modulate the activity and function of other cells to coordinate and control the inflammatory response. It has been suggested that cytokines and chemokines can communicate with sensory nerves through activation of high-affinity receptors, involved in pain and inflammation. Tumor necrosis factor alpha (TNFa), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), Interferon-gamma (IFNγ), CD163 or CD14 are involved in mediating inflammatory response after an
Figure 3: Bar graphs showing TNFα, IL-1β, IL-6 and IFNγ gene expression results after treating Peripheral Blood Mononuclear Cells (PBMC) at 0.0001% and 0.001% concentrations, for 2 hours, and inducing inflammatory response with bacterial LPS, compared to the untreated control. *Represents statistical significance with p value<0.05. **Represents statistical significance with p value<0.01. ***Represents statistical significance with p value<0.001. ****Represents statistical significance with p value<0.0001.

external aggression. For these reasons, the goal of this study is to assess the anti-inflammatory and immunomodulating potential of Liposomal Lactoferrin and Liposomal Zinc. The inflammatory response will be induced by treatment with LPS (Bacterial Lipopolysaccharide), the major constituent of the outer membrane of Gram-negative bacteria and one of the most potent TLR ligands, able to elicit strong immune responses in animals. Regarding protein analysis, results indicated the treatment with bacterial LPS significantly increased CD163 and CD14 protein levels and when treatments with LLF at 0.05% or LLF + LZS at 0.01% and 0.05% concentrations were applied, CD163 protein level was significantly inhibited. For gene expression analysis at 24 hours and 2 hours, results indicated that the induction of gene expression of IFNγ, IL-1β and IL-6 with treatment with LLF or LF + LZS was higher that with LPS. But after 30 minutes of treatment with LPS, TNFα gene expression was significantly decreased by LLF and LLF + LZS treatments at 0.001% concentration.

In conclusion, the results suggest that the treatment with Liposomal Lactoferrin or Liposomal Zinc Sulfate in Peripheral Blood Mononuclear Cells (PBMC), activates a cytokine response mediated by IFNγ, that increases defense mechanisms against infection (induced by LPS). This mechanism seems to be different from that triggered by LPS, precisely because of the specific activation of IFNγ by LLF and LLF + LZS. In our experiment, LPS does not induce IFNγ, however, it does mimic an infection and induces CD163 expression in Peripheral Blood Mononuclear Cells (PBMC), indicating an activation of monocytes/macrophage cells. Liposomal Lactoferrin induces IFNγ, but not TNFα at the concentrations tested. However, addition of Liposomal Zinc to the Liposomal Lactoferrin induces IFNγ and TNFα, which in turn reduce activation of CD163. This may indicate that Liposomal Lactoferrin and Liposomal Zinc helps to counteract the monocyte/macrophage activation induced by infection through activation of IFNγ and TNFα.
Zinc is an important cofactor, signaling molecule with anti-inflammatory activity and antiviral and antioxidant functions. The deficient of zinc in rats may induce increases oxidative stress, induce proinflammatory Cytokines such as TNF-alfa and VCAM-1 expression and induces lung tissue remodeling which may be reversed by zinc supplementation. Zinc can also modulate the viral entry, fusion, replication, viral protein translation and virus budding of respiratory viruses. Zinc also reduces ACE2 activity in rats lungs, inhibit RNA polymerase viral activity by suppressing its replication. Zinc also shortens or reduce respiratory infections by 35% and the recovery of these infections. Liposomal zinc is well absorbed and is the perfect companion to Lactoferrin Doses of liposomal zinc range from 10 to 30 mg. Finally, Zinc is a significant supportive treatment against COVID-19 infections because of its antiviral, antioxidant, anti-inflammatory effect.

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