https://doi.org/10.31925/farmacia.2026.5.21 **ORIGINAL ARTICLE**

EFFECT OF APOPTOZOLE ON CIRCULATING CYTOKINE LEVELS: AN *EX VIVO* WHOLE BLOOD STIMULATION STUDY

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Manuscript received: August 2026

Abstract

Heat shock protein 70 (HSP70) possesses immunomodulatory properties with potential therapeutic relevance. This study investigated the effect of apoptozole (AZL), a novel HSP70 inhibitor, on peripheral blood immune responses. Whole blood from healthy volunteers was challenged *ex vivo* with 1 μg/mL lipopolysaccharide (LPS) or 1 mM acetylsalicylic acid (ASA) with or without 50 μM AZL, for 24 h at 37ºC. Plasma cytokines and soluble immune markers were measured by ELISA. LPS markedly increased TNFα and IL-8, with no significant modification by AZL. ASA significantly increased IL-8, an effect attenuated by AZL co-treatment. In contrast, combined ASA and AZL selectively elevated soluble CD64. No appreciable changes were observed in IL-1β, IL-10, IL-20, or soluble CD206. Collectively, AZL exerted limited effects on LPS-induced responses but selectively modulated IL-8 and soluble CD64 levels under ASA exposure. The context-dependent immunomodulatory effects of AZL on immune cell activation and adhesion warrant further mechanistic investigation.

Rezumat

Proteina de șoc termic 70 (HSP70) prezintă proprietăți imunomodulatoare cu potențial terapeutic. Studiul a evaluat efectul apoptozolului (AZL), un nou inhibitor al HSP70, asupra răspunsului imun la nivelul sângelui periferic. Probele de sânge total recoltate de la voluntari sănătoși au fost stimulate *ex vivo* cu lipopolizaharidă (LPS, 1 μg/mL) sau acid acetilsalicilic (ASA, 1 mM), în prezența sau absența AZL (50 μM), timp de 24 de ore, la 37 °C. Nivelurile citokinelor și ale markerilor imunologici solubili din plasmă au fost determinate prin metoda ELISA. Stimularea cu LPS a determinat o creștere semnificativă a TNFα și IL-8, fără ca AZL să influențeze semnificativ aceste modificări. ASA a produs o creștere semnificativă a IL-8, efect atenuat prin administrarea concomitentă de AZL. În schimb, asocierea ASA cu AZL a determinat o creștere selectivă a nivelului de CD64 solubil. Nu au fost observate modificări semnificative ale IL-1β, IL-10, IL-20 sau CD206 solubil. Rezultatele indică un efect limitat al AZL asupra răspunsului imun indus de LPS, dar evidențiază capacitatea acestuia de a modula selectiv nivelurile IL-8 și CD64 solubil în condițiile expunerii la ASA. Aceste efecte imunomodulatoare, dependente de contextul stimulării, necesită investigații suplimentare pentru elucidarea mecanismelor implicate în activarea și adeziunea celulelor imunitare.

Keywords: apoptozole, HSP70, inflammation, whole blood, cytokines

Introduction

Heat shock proteins (HSPs) were first described in 1962, when Ferruccio Ritossa and colleagues observed that specific gene products in *Drosophila melanogaster* larvae were induced by excessive heat exposure. In 1974, these products were identified and named HSPs (1). HSPs are constantly expressed under physiological anabolic conditions, as they can either function as chaperones or proteases (2). In addition to heat shock, numerous injuries can increase HSP expression rate, including exposure to chemicals, reactive oxygen species, malnutrition, infectious agents, and ischemic injuries (3-7). Furthermore, HSPs play an essential role in the immune response, as they have been detected in host circulation interacting with immune cells and participating in antigen presentation (8-11). Besides their function as immunomodulators, HSPs have been linked to developing autoimmune disorders and some pathological conditions (11, 12).

Beyond their chaperone function, extracellular HSP70 molecules have a remarkable immunomodulatory effect on the innate immune response (11). Extracellular HSP70 can tag tumor cells and mediate their clearance, supporting immunosurveillance (13). In contrast, extracellular HSP70 can trigger a pro-inflammatory immune response by inducing TNF-α release from macrophages (14). One of the main mechanisms that illustrate the dual immunomodulatory effects of HSP70 is its ability to facilitate the access of transcription factor NF-κB to the cell nucleus, promoting gene expression of associated pro-inflammatory cytokines such as IL-6, IL-12, IL-1β, TNF-α and IFN-γ (15). Conversely, the anti-inflammatory effect of HSP70 may relate to its ability to induce IL-10, which limits NF-κB entry into the nucleus (16). The cytoprotective role of

HSP70 has been demonstrated in various autoimmune inflammatory conditions; a study in a mouse arthritis model showed that mycobacterial-based HSP70 (HSP70-B29) promoted Treg proliferation, which significantly restored immune tolerance and controlled inflammation (17). In contrast, other studies reported that HSP70 is implicated in the development of autoimmunity in multiple sclerosis, as it plays a minor role in protecting neural cells from inflammatory injury in an experimental autoimmune encephalomyelitis model (18). Thus, down-regulation or enhanced expression of HSP70 could be a potential immunotherapeutic approach for some pathological conditions.

Apoptozole (AZL;4-((2-(3,5-bis(trifluoromethyl)-phenyl)-4,5-bis(4-methoxyphenyl)-1H-imidazol-1-yl)methyl)benzamide) is a trifluoromethyl-substituted imidazole derivative that specifically targets HSP70 and initiates the intrinsic apoptotic pathway, as AZL renders HSP70 inactive by exclusively binding to its ATPase domain (19). Subsequently, AZL increases DNA fragmentation and reduces mitochondrial membrane potential by targeting HSP70 and HSC70 in lung cancer cell lines P19 and A549 (20). Moreover, it has been demonstrated that AZL can interrupt the association between HSP70 and apoptotic protease activating factor-1, as this interaction is essential for suppressing the caspase-dependent apoptotic pathway (19). In addition, it is evident that AZL targets lysosomal HSP70, as it results in lysosomal membrane permeabilization and leakage of cathepsins into the cytosol, thus triggering apoptotic signaling (21). These findings indicate that AZL mediates cell death *via* caspase-dependent apoptosis; therefore, it is considered a promising anti-tumor agent.

Nevertheless, the role of AZL in mediating whole-blood and peripheral blood mononuclear cell (PBMC) responses to inflammatory insults, including lipopolysaccharides (LPS) and acetylsalicylic acid (ASA), remains unclear. This study aims to investigate the systemic response to AZL in whole blood (WB), which offers a cellular model that mimics the physiological environment encountered *in vivo*. Findings from this study may pave the way to develop immunotherapeutic interventions for inflammatory conditions.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Solarbio Sciences and Technology Co., Ltd. (Beijing, China) unless otherwise noted, including AZL (CAS #1054543-47-3; purity: 99.81%), acetylsalicylic acid (CAS #50-78-2; purity: 99.66%), LPS from *E. coli* O_{111}:B_4, RPMI-1640, and Ficoll-Hypaque. Annexin-V-FITC/propidium iodide was purchased from Biolegend (Biolegend, San Diego, CA, USA). A 10 mM stock solution of AZL and 100 mM of ASA were prepared in dimethyl sulfoxide (DMSO), while 1 mg/mL of LPS was prepared in saline, distributed in aliquots, and stored at -80°C.

Ethical approval and blood collection

The Institutional Review Board of King Saud University approved the study (Project #E-22-7439), in line with the Declaration of Helsinki. Venipuncture collected blood samples from nine healthy volunteers who provided written informed consent into sodium heparin and EDTA vacutainer tubes. Donor characteristics are presented in Table I. Donors were verbally screened and assessed for eligibility by complete blood count (CBC) to exclude inflammatory conditions (Figure 1). Exclusion criteria included age < 18 years, active or recent infection, inflammatory or immunological disorder, or hematological abnormality.

Table I

Characteristics of study subjects

Characteristic	Value
Age, years, range	21-37
Age, years, mean ± SD	29.33 ± 5.80
Age, years, median	28.0
Male, *n* (%)	5 (55.6%)
Female, *n* (%)	4 (44.4%)
Total	9

WB ex vivo stimulation

WB aliquots were diluted 1:2 with PBS and pretreated with 50 μM AZL for 1 h at 37 ℃ in a humidified incubator with 5% CO_2, prior to stimulation with either 1 μg/mL LPS or 1 mM ASA for 24 h in the continued presence of AZL. A total of three independent experiments were conducted, each of which was performed using blood from three subjects. Following treatment, plasma samples from each experiment were collected by centrifugation at 1,000 *x g* for 10 min and stored at -80 ℃ until cytokine analysis (22).

Cytokine detection

Circulating plasma levels of TNF-α (SEKH-0047), IFN-γ (SEKH-0046), IL-1β (SEKH-0002), IL-8 (SEKH-0016), IL-10 (SEKH-0018), IL-20 (SEKH-0031), soluble CD64 (SU-BN12569), and soluble CD206 (SU-BN15005) were measured using Solarbio's ELISA kits following the manufacturer's instructions. Standards and plasma samples were added to microtiter wells pre-coated with specific primary (capture) antibodies against the antigens of interest. They were incubated at 37 ℃ for 90 min, followed by washing to remove excess unbound sample components. Subsequently, 100 μL of biotinylated secondary (detection) antibody was added and incubated at 37 ℃ for 60 min. After washing, add 100 μL of streptavidin-horseradish peroxidase (HRP) conjugate to each well and incubate the plates

at 37 °C for 30 min. Detectable color was developed after the addition of 100 µL of HRP substrate, and 50 µL of sulfuric acid was added to stop the reaction. Absorbance was measured for all cytokines at 450 nm using a Sunrise™ plate reader (Tecan Life Sciences Ltd., Männedorf, Switzerland) (23). Cytokine concentration was interpolated from a standard curve of absorbance *vs*. standard concentration. The four-parameter logistic (4PL) regression was used to calculate the concentration of the unknown samples. Negative interpolated concentrations and values exceeding the upper calibration limit were excluded as unreliable.

CBC

CBC was performed on EDTA whole blood samples using a BC-6200 hematology analyzer (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) to measure white blood cells (WBCs), red blood cells (RBCs), and platelets.

PBMC isolation

WB was diluted 1:2 with PBS and carefully layered over Ficoll-Hypaque. The mononuclear cell layer was isolated by centrifugation at 500 *x g* for 20 min, followed by two washes in PBS at 250 *x g* for 10 min each. Cells were propagated in RPMI-1640 medium under standard cell culture conditions (24).

Apoptosis

PBMC aliquots were stained with annexin-V-FITC (1%) and propidium iodide (PI; 20 µg/mL) in an annexin-V binding buffer containing 0.01 M HEPES, 0.14 M NaCl, and 2.5 mM $CaCl_2$. Cells were incubated in the dark for 10 min at room temperature, and fluorescence was detected on a Northern Lights flow cytometer (Cytek Biosciences, Fremont, CA, USA) with 488 nm excitation. Annexin-V-FITC fluorescence was captured in the green emission channel at 520 nm, whereas PI fluorescence was collected in the orange-red emission channel at 620 nm. Unstained and single-stained controls were included for spectral unmixing, and gating excluded noncellular debris while retaining apoptotic populations. A total of 10,000 events were acquired *per* sample (25).

Statistical analysis

Results are shown as means ± SD. Experiments generated data from nine independent donors across three experimental runs (three donors *per* run). Means were analyzed using one-way ANOVA followed by Tukey's or Dunnett's post-hoc tests as appropriate. All statistical analyses were performed by GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA) with statistical significance set at $P < 0.05$.

Results and Discussion

Lack of cytotoxic effects by AZL, LPS, and ASA

First, we confirmed that the concentrations of AZL, LPS, and ASA used were not cytotoxic to WB (Figure 1) or PBMCs (Figure 2).

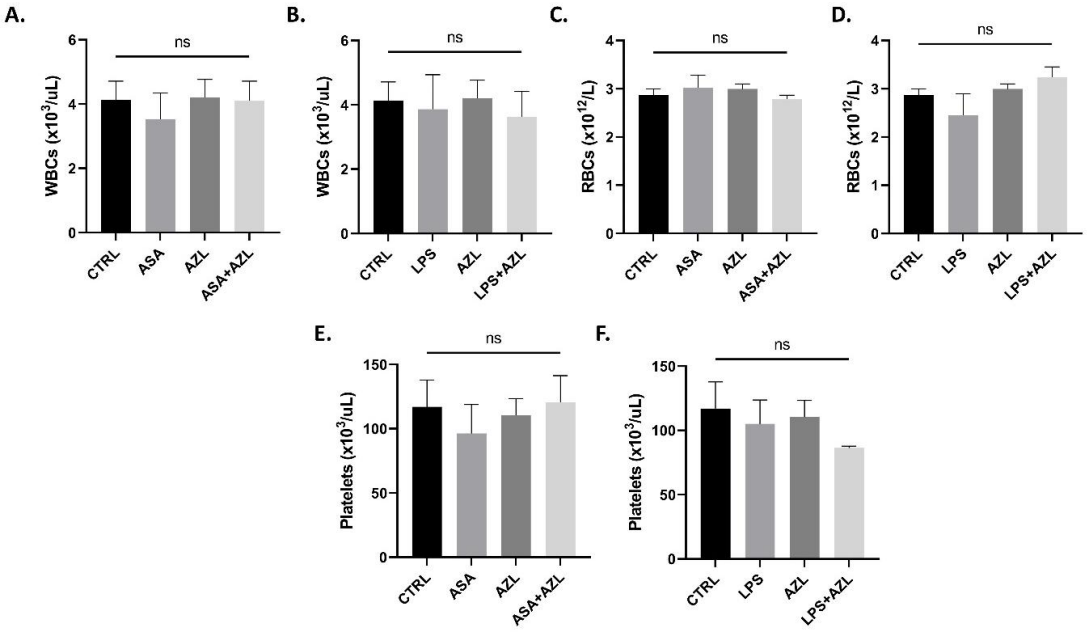


Figure 1.

AZL is not cytotoxic to WB. WBC count under (A) ASA and (B) LPS stimulation. RBC count under (C) ASA and (D) LPS stimulation. Platelet count under (E) ASA and (F) LPS stimulation. WB was incubated for 24 h at 37 °C with ASA (1 mM) or LPS (1 µg/mL), with and without pretreatment for 1 h with AZL (50 µM). Results are shown as means ± SD (n = 9), analyzed by one-way ANOVA followed by Dunnett's test.

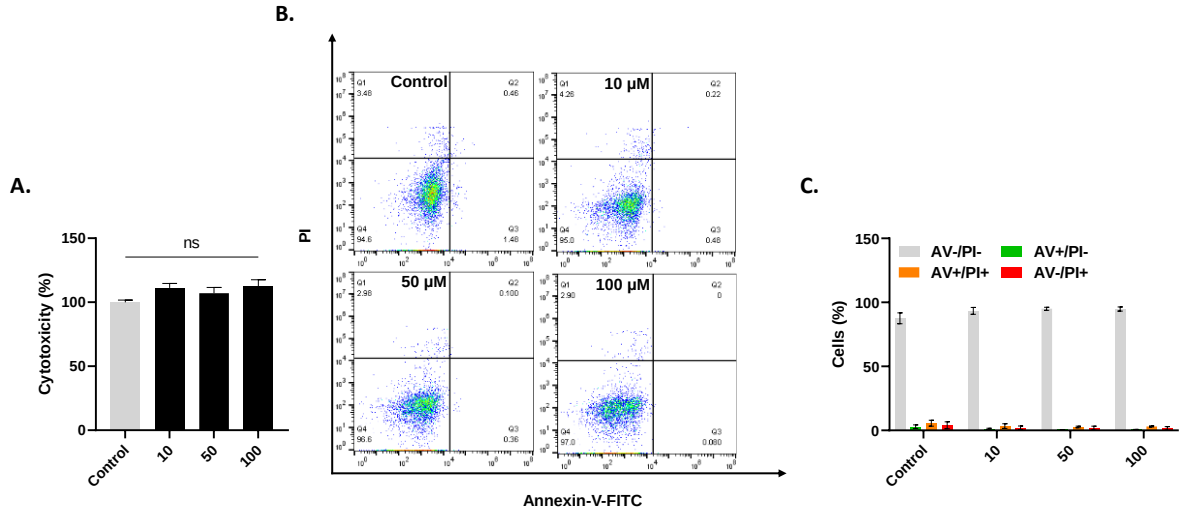


Figure 2.

AZL is not cytotoxic to PBMCs. (A) Cytotoxicity of AZL in PBMCs. (B) Representative dot plots of annexin-V-FITC and PI. (C) Percentage of apoptotic and necrotic cells. PBMCs were exposed to 10, 50, or 100 µM AZL for 24 h at 37 °C. Results are shown as means ± SD (n = 9), analyzed by one-way ANOVA followed by Dunnett's test.

Differential effects of AZL on TNFα and IFNγ responses

Stimulation with LPS markedly increased TNFα secretion compared with the untreated control (Figure 3A). Co-treatment with AZL did not significantly modify the LPS-induced TNFα response. Similarly, neither ASA alone nor in combination with AZL significantly altered TNFα concentrations relative to the control group. In contrast, IFNγ levels were not significantly different across all experimental conditions (Figure 3B). Although inter-individual variability was evident, neither LPS nor ASA, with or without AZL, produced significant changes compared to the control samples.

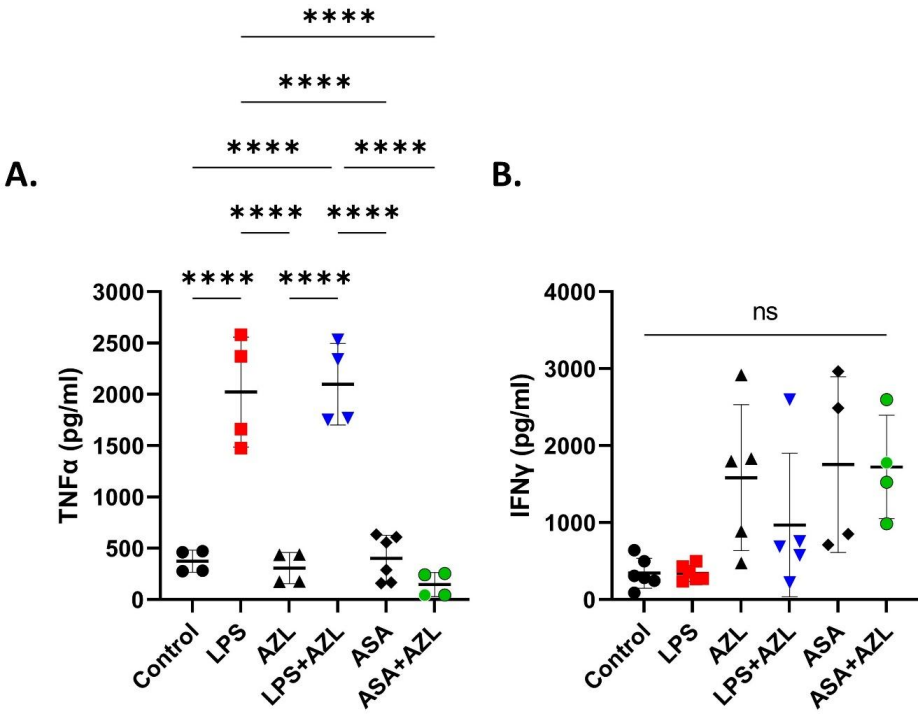


Figure 3.

Effects of AZL on TNFα and IFNγ secretion. (A) Plasma TNFα concentrations. (B) Plasma IFNγ concentrations. Whole blood was incubated for 24 h at 37 °C with AZL (50 µM) alone or in combination with LPS (1 µg/mL) or ASA (1 mM). results are presented as individual donor values with means ± SD (*n* = 4-6). Statistical analysis was performed using one-way ANOVA with Tukey's correction.

AZL selectively suppresses ASA-induced IL-8 but not IL-1β

As shown in Figure 4A, IL-1β concentrations did not differ significantly across experimental groups. However, IL-8 exhibited a distinct response pattern (Figure 4B). Both LPS and ASA significantly increased IL-8 concentrations compared with untreated controls. AZL did not significantly attenuate spontaneous or LPS-induced IL-8 response. Nonetheless, co-treatment with AZL significantly suppressed ASA-induced IL-8 secretion to levels comparable to the control group.

Anti-inflammatory cytokines show no significant differences across groups

No statistically significant differences were observed in IL-10 concentrations among the experimental groups (Figure 5A). Likewise, IL-20 showed considerable donor-to-donor variation with no significant alterations by AZL, LPS, ASA, or their combinations (Figure 5B).

AZL modulates soluble CD64 but not CD206 concentrations

Soluble CD64 plasma levels differed significantly among treatment groups (Figure 6A). In particular, co-treatment with ASA and AZL resulted in significantly higher circulating CD64 concentrations than in the control and AZL groups. LPS did not affect CD64 irrespective of AZL availability. No significant differences were found in soluble CD206 levels across all treatment groups (Figure 6B), indicating that neither inflammatory stimulus nor AZL appreciably affected its release in plasma under the present experimental conditions.

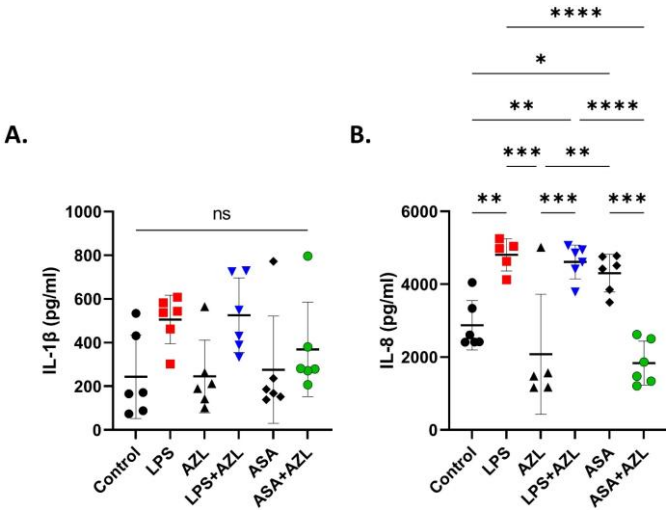


Figure 4.

Effects of AZL on IL-1β and IL-8 secretion. (A) Plasma IL-1β concentrations. (B) Plasma IL-8 concentrations. Whole blood was incubated for 24 h at 37 °C with AZL (50 μM) alone or in combination with LPS (1 μg/mL) or ASA (1 mM). Results are presented as individual donor values with means ± SD (n = 4-6). Statistical analysis was performed using one-way ANOVA with Tukey's correction.

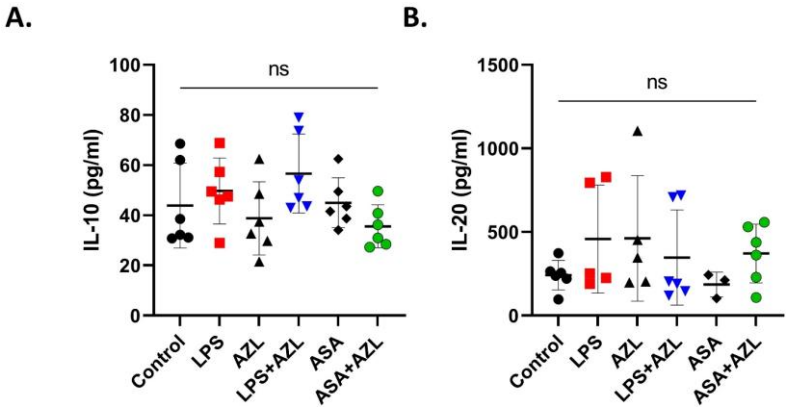


Figure 5.

Effects of AZL on IL-10 and IL-20 secretion. (A) Plasma IL-10 concentrations. (B) Plasma IL-20 concentrations. Whole blood was incubated for 24 h at 37 °C with AZL (50 μM) alone or in combination with LPS (1 μg/mL) or ASA (1 mM). results are presented as individual donor values with means ± SD (n = 4-6). Statistical analysis was performed using one-way ANOVA with Tukey's correction.

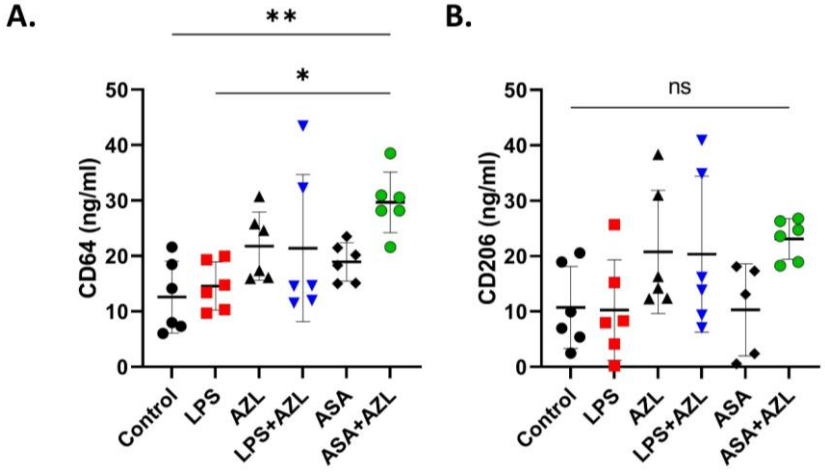


Figure 6.

Effects of AZL on soluble CD64 and CD206 levels. (A) Plasma CD64 concentrations. (B) Plasma CD206 concentrations. Whole blood was incubated for 24 h at 37 °C with AZL (50 μM) alone or in combination with LPS (1 μg/mL) or ASA (1 mM). Results are presented as individual donor values with means ± SD (n = 4-6). Statistical analysis was performed using one-way ANOVA with Tukey's correction.

The WB *ex vivo* stimulation assay provides an optimal milieu to investigate chemokine and cytokine responses under conditions that closely resemble physiological conditions (22,26). In this pilot study, we examined, for the first time, the immunomodulatory effect of AZL on WB exposed to ASA and LPS. This approach combines LPS-induced bacterial inflammatory stimulation with ASA-mediated pharmacological modulation of cyclooxygenase (COX) and NF-κB-associated signaling. It should be noted that the measured cytokines in this exploratory study reflect soluble plasma forms, and, as such, may not directly correspond to surface expression, receptor density, or downstream signaling at the cellular level. Furthermore, the relatively small sample sizes and interindividual variability may have limited the detection of treatment-associated differences, particularly for IFN-γ, IL-1β, IL-10, IL-20, and CD206. Similarly, the absence of a T-cell stimulus may have constrained IFN-γ responses under the experimental conditions. Nevertheless, unlike flow cytometric phenotyping, which typically requires cell isolation, antibody staining, gating, and analytical processing, the WB model directly assesses the systemic, multicellular immune response and is readily adaptable to routine clinical laboratory workflows under minimally manipulated conditions.

This study exploited AZL treatment *ex vivo* in the presence of LPS, which did not significantly attenuate LPS-induced TNF-α release. HSP70 administration has been shown to prevent and ameliorate inflammatory damage in experimental disease models (27), consistent with AZL's role as an HSP70 inhibitor. Multiple studies have also depicted the role of HSP70 in attenuating inflammatory responses both *in vivo* and *in vitro*. A study of HSP70-deficient mice showed increased mortality compared with wild-type mice in a septic shock model (28); in addition, HSP70 halts LPS-induced NF-κB activation and pro-inflammatory cytokine release (29). The role of NF-κB in regulating the expression of pro-inflammatory genes mediated by inflammation is evident. NF-κB proteins exist in an inactive state; when exposed to stimuli, the NF-κB complex, mainly p50/p65, is phosphorylated and translocated into the nucleus, which mediates the upregulation and expression of pro-inflammatory genes by binding to their promoter sites (30,31).

HSP70 overexpression could downregulate pro-inflammatory mediator production by blocking NF-κB activation, thereby alleviating the inflammatory state (32,33). A study investigating the anti-inflammatory effect of Avenanthramide (AVA) on small intestine damage in an ovalbumin-induced food allergy model showed that AVA administration mitigated inflammation and lowered serum concentrations of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-25, and IL-33 (33,34). Moreover, AVA has upregulated the expression of HSP70 and inhibited the phosphorylation of NF-κB. To validate the involvement of Hsp70-NF-κB signaling, AZL was added, as it diminished the anti-inflammatory effects of AVA in the model.

Furthermore, our study shows that AZL did not significantly attenuate LPS-induced TNF-α release. This finding is broadly consistent with previous reports demonstrating that AZL counteracted the anti-inflammatory effects of AVA (33,34), although these studies investigated different experimental conditions. Notably, the same was reported for IL-8 levels, as HSP70 inhibition had negligible influence on its immune modulation. Nonetheless, HSP70 could have a dual effect by aggravating the pro-inflammatory response and acting synergistically with

LPS (35). It was shown that extracellular HSP70 (eHSP70) can serve as a damage-associated molecular pattern (DAMP), thus activating monocytes and macrophages via binding to Toll-like receptor 4 (TLR4), further stimulating cytokine production and propagating the inflammatory response through MyD88/IRAK/NF-κB signal transduction pathway activation (36,37). Subsequently, TLR4 activation could induce NF-κB and MAPK Kinase pathways, mediating the synthesis and release of TNF-α, IL-8, IL-6, IL-1, and IL-10 (38-40).

In addition, it has been revealed that recombinant human HSP70 (rhHSP70) is implicated in the inflammatory reaction in chronic obstructive pulmonary disease patients (35). Using the monocytic THP-1 cell line as a cellular model, rhHsp70 alone induced IL-1α and IL-8 release, while combined treatment with LPS produced a synergistic inflammatory response. However, treatment with rhHsp70 alone did not induce IL-6 and TNF-α, as this is consistent with a previous study (37), which elucidated the downregulation of TNF-α gene expression upon treatment with HSP70, albeit in primary monocytes, not THP-1 cells. Conversely, the immunomodulatory effect of eHSP70 could be subjected to certain negative feedback mechanisms (35). It was shown in many studies that eHSP70 could render endotoxin tolerance if low concentrations were added before LPS challenge, as well as offering protection against LPS in rats with sepsis (41-44).

A major finding of our study is that AZL selectively inhibits ASA-induced IL-8. The main mechanism of action of ASA was originally thought to be suppression of endogenous prostaglandin synthesis via inhibiting COX activity (45,46). However, studies have shown that ASA has other COX-independent immunomodulatory mechanisms, including NF-κB pathway inhibition (47), stimulation of nitric oxide release (48), and lipoxin synthesis (49). Moreover, ASA exhibits a dual immunomodulatory response that is dose-dependent. It has been previously described that COX-2 induces the expression of both pro-inflammatory and anti-inflammatory prostaglandins, suggesting a complex role in the inflammatory cascade (50). A study exploiting the stimulatory effect of ASA in whole blood reported an elevated production of IL-2 and IFN-γ at 0.1 mM ASA exposure, rendering a stimulatory effect on IL-6 and IL-8 synthesis upon addition of 1.0-5.0 mM ASA (51). In the current study, we used 1.0 mM ASA, which corresponds to the concentration range used in previous studies (51,52). Moreover, therapeutic plasma salicylate concentrations, the principal ASA metabolite, can exceed 3.0 mM (53). Although this concentration cannot be directly equated to that of the parent compound, it nonetheless demonstrates the clinical relevance of millimolar salicylate exposure.

Regarding IL-8 levels, only a 20-30% decline was observed at an ASA concentration of 15 mM. Notably, the levels of IL-8 were barely diminished by ASA in this whole blood model. Additionally, a study investigating the inflammatory effects of cigarette smoke extract (CSE) and its combination with or without eHSP70 on monocyte-derived macrophages (MDMs) and the THP-1 cell line showed that eHSP70 stimulates cytokine production via TLR2 and TLR4 and activates the NF-κB and MAPK pathways (54). In THP-1 cells, however, a combination of both CSE and rhHSP70 did not result in a significant difference in IL-8 production compared to CSE alone. Conversely, when both CSE and rhHSP70 were applied in MDMs, the combination caused a synergistic increase in IL-8 secretion, potentially due to the overexpression of the TLR2 gene present in MDMs after the combined exposure to CSE and rhHSP70. Nonetheless, in THP-1, these effects on IL-8 production may not reflect TLR2 or TLR4 expression and could be mediated by another eHSP70 receptor. Both TLR2 and TLR4 receptors are found on alveolar macrophages, monocytes, and neutrophils (55). Upon stimulation, this leads to the activation of MyD88/IRAK/NF-κB and MAPK signaling pathways. Additionally, in inhibition experiments, it was evident that JNK contributed to IL-8 secretion in MDMs, while the underlying mechanism in THP-1 cells involved both ERK and JNK activation. These findings showcase the dual immunomodulatory effects of HSP70, which depend on overall inflammatory context and the cell type (56,57). Thus, our findings provide valuable data for future investigations concerning the therapeutic aspect of apoptozole in inflammatory conditions.

Nonetheless, the levels of IFN-γ, IL-1β, IL-10, IL-20, and CD206 remain not significantly different upon exposure to either LPS or ASA. Intriguingly, another finding of this study is that AZL aggravated plasma CD64 levels when combined with ASA. This is particularly relevant to inflammatory conditions such as Crohn's disease and rheumatoid arthritis (58,59). Also, as a critical biomarker of sepsis, cellular CD64 is strongly upregulated in response to proinflammatory cytokines and plays a role in both innate and adaptive immune responses during sepsis pathogenesis (60). Having a dual immunoinflammatory role, ASA indeed could contribute to augmenting or ameliorating an underlying inflammatory response. A study found that upon analyzing peripheral leukocytes using a microarray assay, there was significant hyperactivity of TH17 immunity in sepsis patients (61). In particular, innate immunity genes were markedly upregulated, including HSP70 protein expression, neutrophil elastase, caspases, and IgG and IgA Fc receptors (CD64, CD32). Therefore, the immune-protective role of HSP70 could be essential in mitigating the pathological manifestations

in sepsis. A review exploiting this speculation has concluded that HSP70 exerts a protective effect intracellularly, and its decline, thus accumulating in the extracellular space, is a biomarker of sepsis severity (62).

Conclusions

In conclusion, this work presents evidence of the immunomodulatory role of AZL in whole blood and highlights the shared and distinct effects with ASA in response to LPS challenge. These findings support the potential clinical utility of AZL for inflammatory regulation in non-infectious conditions and its role in preserving antibacterial immune signatures. Future studies should interrogate the mechanisms and pathways through which AZL interferes with ASA but not LPS signaling, in addition to cell-specific functional profiling and translational validation in patients with inflammatory conditions.

Acknowledgement

The authors acknowledge the financial support provided by the Ongoing Research Funding program (ORF-2026-554), King Saud University, Riyadh, Saudi Arabia.

Funding

This work was funded by the Ongoing Research Funding program, King Saud University, Riyadh, Saudi Arabia under grant number ORF-2026-554.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, M.A.A., upon reasonable request.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Review Board of King Saud University (Project #E-22-7439/2022), in line with the Declaration of Helsinki. Informed consent was obtained from all patients included in the study.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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