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Original Article

Galectin-9 induces IL-1β production as a key inflammatory cytokine in the acute myeloid leukemia cell line (U937)

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Abstract

Background and purpose: T-cell immunoglobulin and mucin-domain containing protein-3 (TIM-3)/ galectin-9 (Gal-9)/ autocrine loop in myeloid leukemia stem cells provokes inflammation through the NF-κB signaling pathway, which is influential in the expression of inflammatory factors. Interleukin1β (IL-1β) is a vital inflammatory cytokine that plays an important role in the proliferation and therapy resistance of acute myeloid leukemia (AML) cells. This study aimed to assess the effect of Gal-9 on IL-1β in the human leukemic U937 cell line.

Experimental approach: The U937 cells were cultured in different concentrations of Gal-9. Cell counting kit-8 was used to assess the effect of Gal-9 on human leukemic U937 cell proliferation. Also, its impact on the expression of *TIM-3, Gal-9, IL-1β, IL-1βR, IL-1βRAP,* and *NLRP3* genes and IL-1β protein was studied by RT-PCR and ELISA, respectively. Moreover, the effect of Gal-9 on the NF-κB signaling pathway was evaluated by western blotting.

Findings/Results: U937 cells were expanded in the presence of Gal-9 in a concentration-dependent manner. Following treatment of U937 cells with Gal-9, the gene expression of *Gal-9*, *IL-1B*, *IL-1BR*, and *IL-1BRAP* were significantly upregulated compared to the control group. The IL-1β concentration increased following Gal-9 treatment in a concentration-dependent manner, while following time-pass its level significantly decreased. Furthermore, Gal-9 slightly increased NF-κB phosphorylation.

Conclusion and implications: Gal-9 increased IL-1β level as a critical inflammatory cytokine in the proliferation and resistance of AML cells to therapy. According to this finding, targeting and blocking the TIM-3/Gal-9 autocrine loop can suppress IL-1β production and facilitate AML treatment.

Keywords: AML; GAL-9; Inflammation; IL-1β, NF-κB.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous malignancy caused by abnormal clonal expansion of myeloid progenitor cells associated with differentiation arrest in the bone marrow. AML is the most prevalent type of acute leukemia in adults arising from genetic alterations in hematopoietic progenitor cells, known as *de novo* AML, or can be secondary due to exposure to toxic agents or as the result of other malignancies, including myelodysplastic syndromes or myeloproliferative neoplasms (1). Also, it can result from treatment of an unrelated disease, including solid tumors, other hematologic malignancies (for instance, Hodgkin disease, non-Hodgkin lymphoma, and multiple myeloma), or autoimmune disorders by chemotherapy or radiotherapy, which is known as therapy-related AML (2).

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The heterogeneity of underlying causes can affect both clinical manifestation and treatment procedure. Administration of cytarabine along with anthracyline is considered the main chemotherapy regimen for AML therapy, which has not been successful so far (3-5).

Galectin-9 (Gal-9) is a β-galactoside-binding lectin with two carbohydrate recognition domains expressed by various cells and tissues at different extents (6,7). Gal-9 is highly secreted by myeloid leukemia cells and has an influential effect on the expansion of leukemic cells through the essential T-cell immunoglobulin and mucin-domain containing protein-3 (TIM-3)/Gal-9 autocrine loop, leading to the activation of several vital signaling pathways, proliferation of cancer cells, and suppression of immune response (8-10). As a versatile molecule, Gal-9 participates in several processes, including cell growth, cell differentiation, cell aggregation, mitochondria-mediated apoptosis, chemoattraction, migration, and immunomodulation, based on its localization in the nucleus, cytoplasm, or extracellular space (11,12). However, Gal-9 activity is extremely controversial in the immune system and can be bilateral. Specifically, Gal-9 can suppress adaptive immunity *via* different mechanisms, such as apoptosis of T helper-1 cells and differentiation of regulatory T cells (13). On the other hand, it can provoke innate immunity and inflammation through the recruitment of innate immune cells, stimulating the maturation of dendritic cells and the secretion of pro-inflammatory cytokines and chemokines (14–18). It is worth mentioning that Gal-9 can induce nuclear factor-κB (NF-κB) signaling pathway following binding to its receptor called TIM-3, which is expressed on AML leukemic stem cells but not normal hematopoietic stem cells (9).

NF-κB is an indispensable transcription factor controlling different facets of innate and adaptive immunity in most inflammatory responses. NF-κB can induce and regulate inflammation by regulating the expression of multiple target genes, including pro-inflammatory cytokines, chemokines, and adhesion molecules (19-21). Among inflammatory cytokines, interleukin-1β (IL-1β) is considered a critical cytokine in leukemia, and its functionality is mediated through IL-1 receptor (IL-1R) signaling, which is composed of two components, including IL-1β receptor (IL-1βR) and IL-1βR accessory protein (IL-1βRAP). IL-1β can augment the survival and proliferation of AML cells through the p38 mitogen-activated protein (MAP) kinases (P38MAPK) signaling pathway in a way that if the signaling is blocked by a monoclonal antibody, expansion of leukemic cells will get blocked and normal hematopoiesis will be retrieved. In addition, IL-1β can trigger AML resistance to apoptosis *via* promoting anti-apoptotic molecules such as B-cell lymphoma 2 (Bcl-2) and suppressing pro-apoptotic molecules such as Bcl-2-associated death promoter (22-25). Considering all the above-mentioned mechanisms, in this study, we selected U973 as a human leukemic cell line to assess the effect of Gal-9 on the inflammatory pathway in these cells by evaluating IL-1β-related gene expression and IL-1β production.

MATERIALS AND METHODS

Materials

The human leukemic U937 cell line was purchased from the Stem Cell Technology Research Center of Iran (Tehran, Iran). Cell-counting Kit-8 (CCK-8) and recombinant human Gal-9 (rhGal-9) were purchased from BioLegend, San Diego, CA, USA. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich. TrizoLEX reagent was provided from DNA Biotech, Tehran, Iran, and an Easy cDNA Synthesis Kit was provided from Parstous Biotechnology, Mashhad, Iran. Real-time polymerase chain reaction (RT-PCR) master mix was provided from Biofact, Daejeon, South Korea.

Culture of U937 cell line

The U937 cells were cultured in a complete medium of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The following experiments were conducted during the logarithmic growth stage of the cells.

U937 cell proliferation assay

CCK-8 was used to determine the effect of Gal-9 on U937 cell proliferation. Briefly, 1 × 10^5 U937 cells were seeded per well in 96-well plates and primed with different concentrations of rhGal-9 (3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 ng/mL) for 24 h. At the end of the incubation time, 10 µL of CCK-8 solution was added to each well, which led to the production of a water-soluble formazan dye, proportional to viable cell numbers. The plate was incubated for three hours, and finally, the absorbance was recorded at 460 nm using a microplate reader (BIOHIT BP800, United Kingdom).

Assessment of TIM-3, Gal-9, IL-1β, IL-1βR, IL-1βRAP, and NLRP3 gene expression

RT-PCR was carried out to assess the effect of Gal-9 on the expression level of numerous target genes related to IL-1-mediated inflammation, including *TIM-3, Gal-9, IL-1β, IL-1βR, IL-1βRAP,* and *NLR* family pyrin domain-containing 3 (*NLRP3*). In detail, 1.5 × 10^6 U937 cells were seeded in 6-well plates, stimulated with 100 ng/mL of LPS and various concentrations of rhGal-9, and incubated for 24 h. For the time-dependent study, cells were stimulated with 50 ng/mL of rhGal-9 and incubated for varying intervals (6-48 hrs). Total RNA was extracted using TrizoLEX reagent through the guanidinium thiocyanate-phenol-chloroform extraction method. The quality of the extracted total RNA was determined with a NanoDrop 2000c spectrophotometer and 1 µg/mL of total RNA was applied for cDNA synthesis according to the Easy cDNA Synthesis Kit manual. RT-PCR was performed based on the 2x SYBR-Green master mix instructions. Following 1 cycle of 95 °C for 15 min, three-step thermal cycling consisting of 40 cycles of 20 s at 95 °C, 30 s at 58-60 °C, and 30 s at 72 °C was repeated. Table 1 shows the sequences of the applied primers. *GAPDH* was considered the housekeeping gene and the $2^{-(\Delta\Delta CT)}$ method was used to calculate the relative expression of the target genes.

Measurement of IL-1β protein in cell lysate

IL-1β protein was measured by the ELISA method using the Human IL-1β ELISA development kit. The ELISA procedure was performed according to the manufacturer's instructions. In brief, 1 × 10^6 U937 cells were seeded per well in 24-well plates and treated with LPS (100 ng/mL) and different concentrations of rhGal-9 for 24 h, or alternatively treated with LPS (100 ng/mL) and rhGal-9 (50 ng/mL) for varying intervals. After incubation time, cell lysates were collected to evaluate the amount of IL-1β protein.

Measurement of total and phosphorylated NF-κB

Western blotting was carried out to evaluate the effect of rhGal-9 on the total and phosphorylated form of NF-κB. Approximately 3 × 10^6 cells were seeded per well in 6-well plates and treated with LPS (100 ng/mL) and rhGal-9 (50 ng/mL) for 24 h. Then, cells were harvested, washed with cold phosphate-buffered saline (PBS) once, and lysed with radioimmunoprecipitation assay buffer (RIPA; Santa Cruz Biotechnology, Dallas, Texas, USA), containing protease and phosphatase inhibitor cocktail. The protein concentration in the whole cell lysates was measured by a bicinchoninic acid kit (DNA Biotech, Tehran, Iran), and then equal protein concentrations (70 µg) were loaded on 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The resolved proteins were electro-transferred to the polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk in PBS containing 0.2% tween 20 (PBST), for 2 h at room temperature. The membrane was then incubated with mouse anti-human NF-κB p65 (1:200, Santa Cruz Biotechnology, Dallas, Texas, #sc-514451), mouse anti-human pNF-κB p65 (1:200, Santa Cruz Biotechnology, Dallas, Texas, #sc-136548), and rabbit anti-human β actin (1:1000, Abcam, Cambridge, UK, #ab8227) antibodies diluted in 2% skimmed milk/PBST at 4 °C overnight, followed by three washes (each 5 min) in PBST. In continue, the membrane was incubated with appropriate dilutions of secondary antibodies (HRP-conjugated goat anti-mouse IgG,1:2000, BioLegend, San Diego, CA, USA, #405306 and HRP-conjugated goat anti-rabbit IgG, 1:1000, Sigma-Aldrich, Germany, #A 6154) for 1 h at room temperature, followed by 4 washes with PBST. Final visualization was performed using an enhanced chemiluminescence reagent (Intron Biotechnology, Seongnam-Si, South Korea).

Table 1. The primers' sequences applied in Real-time polymerase chain reaction.

Target gene name	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: TGGTGAAGACGCCAGTGGA	58	138
TIM-3	Forward: TCTTCCCTTTGACTGTGTCCT Reverse: TTCAAACACAGGACAGGCTC	60	175
Gal-9	Forward: GATGAGAATGCTGTGGTCCG Reverse: GAAGCCGCCTATGTCTGCA	58	260
IL-1β	Forward: AGCTTGGTGATGTCTGGTCC Reverse: ACGCAGGACAGGTACAGATT	58	167
IL-1βR	Forward: GTGCTTTGGTACAGGGATTCCTG Reverse: CACAGTCAGAGGTAGACCCTTC	60	121
IL-1βRAP	Forward: CGGGCTCATTTTGGAACAGAT Reverse: GGCAGACTGTCTCGGTCAA	60	170
NLRP3	Forward: ATGAGCCGAAGTGGGGTTCAG Reverse: GAGACGCAGTCGTGTGTAG	58	251

TIM-3, T cell immunoglobulin domain and mucin domain-3; Gal-9, galectin-9; IL, interleukin; NLRP3, NLR family pyrin domain containing 3.

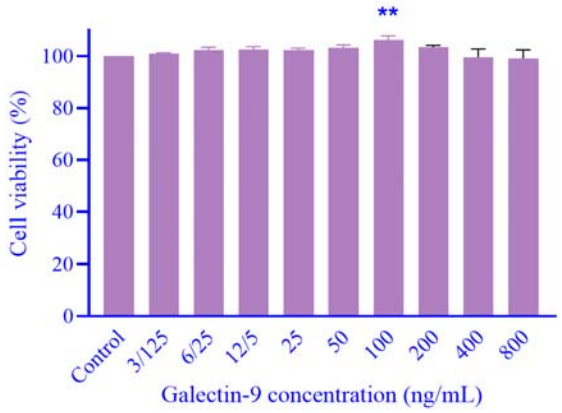

Fig. 1. The effect of Gal-9 on the proliferation of U937 cells. U937 cells were treated with various concentrations of rhGal-9 and the proliferation of the cells was measured by CCK-8 after 24 h. The data were achieved through two independent experiments; each was performed in triplicate. Data are represented as the mean ± SD. $^{**}P < 0.01$ indicates significant differences compared to the control group. rhGal-9, recombinant human galectin-9; CCK-8, Cell-counting Kit-8.

Statistical analysis

Data were analyzed using Graph Pad Prism software 9 (San Diego, CA, USA). One-way ANOVA and two-way ANOVA followed by Bonferroni-Dunn and Tukey`s multiple comparison post-hoc tests were performed to compare more than two independent groups in concentration-dependent and time-dependent studies, respectively. The results are expressed as the mean ± SD. Western blot results were analyzed by nonparametric tests. Kruskal-Wallis, followed by the Dunn test was performed to compare different independent groups. Moreover, the Benjamini-Hochberg test was done to control the false discovery rate (FDR) in multiple testing experiments. Data are presented as median ± interquartile range (IQR). *P*-values < 0.05 were considered statistically significant.

RESULTS

The impact of Gal-9 on the proliferation of U937 cells

CCK-8 assay revealed that rhGal-9 provoked the proliferation of U937 cells in a concentration-dependent manner through a gradual increase in rhGal-9 concentration to a certain extent of 100 ng/mL. Although the higher concentrations of rhGal-9 (> 100 ng/mL) did not induce cell proliferation compared to the control group, significant cytotoxicity was not seen either (Fig. 1).

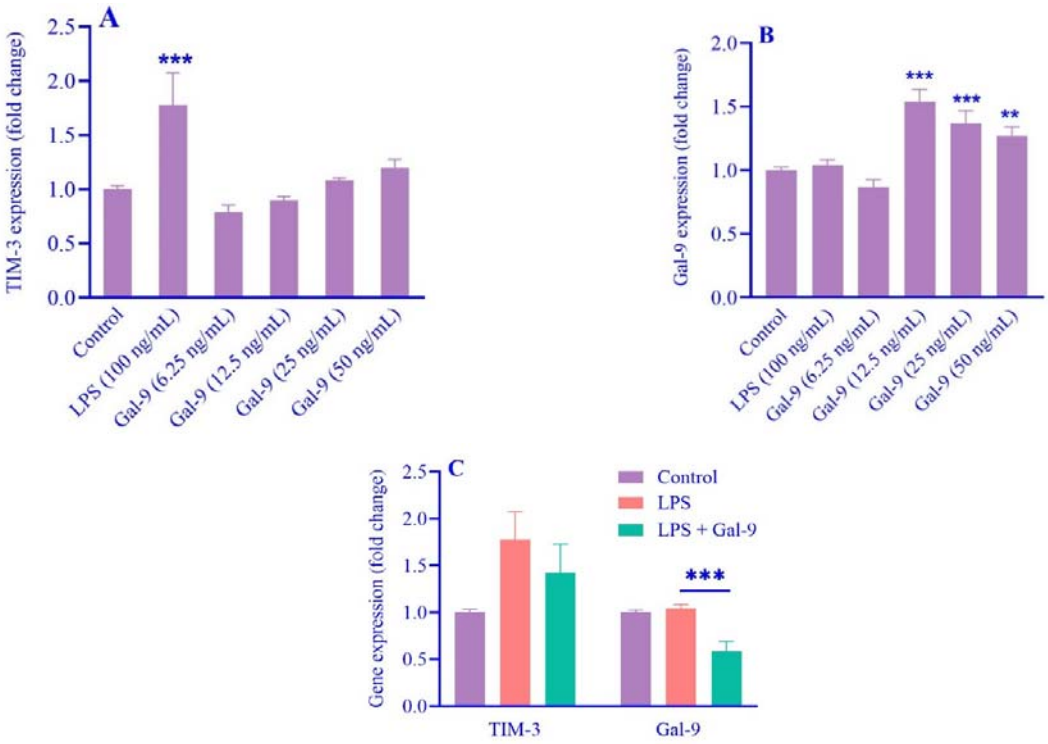

Fig. 2. The effect of (A and B) Gal-9 alone or (C) in combination with LPS on *TIM-3* and *Gal-9* gene expression of U937 cells. Different concentrations of rhGal-9 alone or in combination with LPS were added to U937 cells and after 24 h incubation, the *TIM-3* and *Gal-9* gene expression were studied by RT-PCR. The data were achieved through two independent experiments, each performed in triplicate. Data are represented as the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the control group and $^{###}P < 0.001$ between the designated groups. Gal-9, Galectin-9; LPS, lipopolysaccharide; *TIM-3,* T cell immunoglobulin domain and mucin domain-3.

Gal-9 reinforced the TIM-3/GAL-9 autocrine loop by promoting Gal-9 expression

The gene expression study showed that although there was an upward trend in TIM-3 expression following the treatment of the U937 cells with different concentrations of rhGal-9, this molecule did not significantly increase its receptor expression compared to the control, particularly at low concentrations (Fig. 2A). On the other hand, rhGal-9 significantly augmented its own expression in comparison with the control in all applied concentrations except for the lowest concentration (6.25 ng/mL; Fig. 2B). Furthermore, in the rhGal-9 and LPS co-treatment group, expression of both TIM-3 and Gal-9 decreased compared to the merely LPS-treated group. Gal-9 expression was significantly reduced in the co-treated group, but the reduction of TIM-3 expression was not statistically significant (Fig. 2C).

The effect of Gal-9 on the key inflammatory cytokine IL-1β

Evaluation of gene expression revealed that rhGal-9 was able to significantly increase IL-1β expression in a concentration-dependent manner in comparison with the control (Fig. 3A). In the time-dependent study, there was a steady increase in IL-1β expression in the treated cells at 24 h and 48 h in comparison with the 6-h treated group (Fig. 3B). At the protein level, the results of the concentration-dependent study showed that rhGal-9 significantly increased the amount of IL-1β protein compared to the control, although no significant difference among various concentrations of rhGal-9 was observed (Fig. 3C). Time-dependent study revealed a significant steady reduction of IL-1β protein level in the 24-h and 48-h treated groups compared to the 6-h treated group (Fig. 3D).

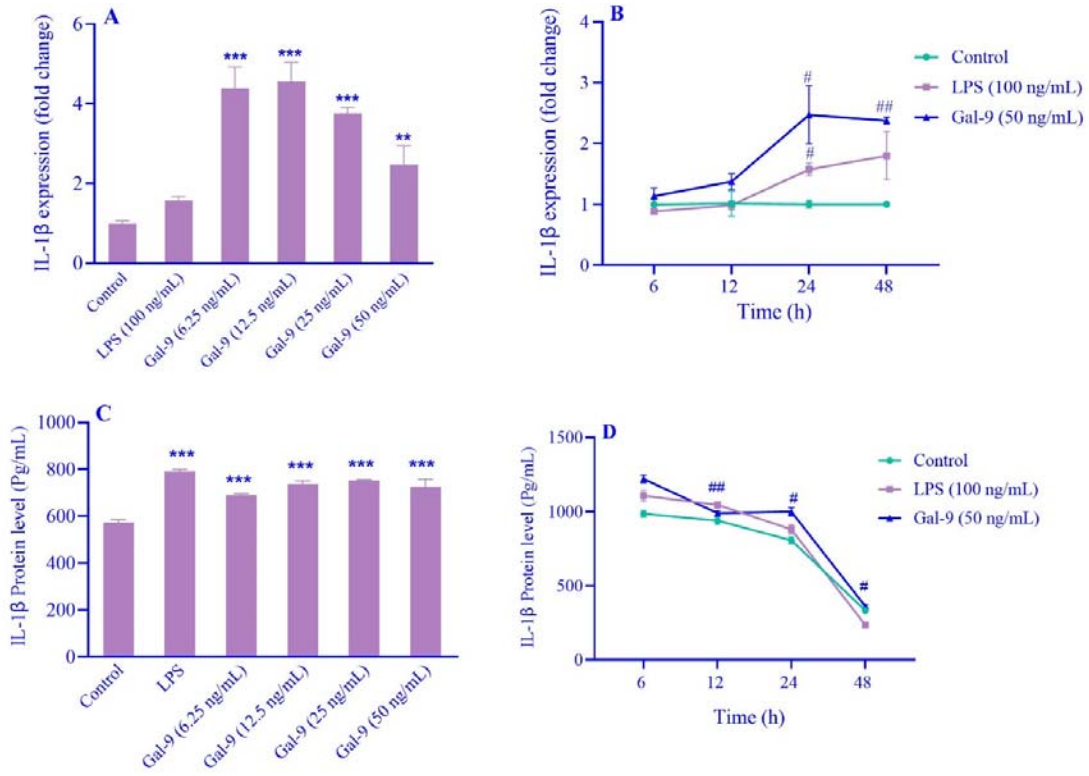

Fig. 3. The effect of Gal-9 on *IL-1β* gene and protein expression of U937 cells. U937 cells were treated with different concentrations of rhGal-9 or a determined concentration of rhGal-9 and then *IL-1β* gene and protein expression levels were measured by RT-PCR and ELISA methods, respectively. In the concentration-dependent study, the effect of various concentrations of rhGal-9 on *IL-1β* (A) gene and (C) protein expression was studied after 24 h treatment, and in the time-dependent study, the effect of a determined concentration of rhGal-9 on *IL-1β* (B) gene and (D) protein expression was studied following 6, 12, 24, and 48 h treatment of the cells. The data were obtained through two independent experiments, each performed in triplicate. Data are represented as the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the control group and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the 6-h treatment groups. Gal-9, Galectin-9; LPS, lipopolysaccharide; IL, interleukin.

Moreover, in the co-treatment group, not only the gene expression of IL-1β but also its protein level was significantly reinforced compared to the merely LPS-treated group (Fig. 4A and B).

The impact of Gal-9 on IL-1β signaling through its receptor expression

The results demonstrated that different concentrations of rhGal-9, except for the lowest concentration (6.25 ng/mL), significantly increased the expression of IL-1βR in comparison with the control. It looked as if there was a gradual upward increment pattern among different concentrations of rhGal-9, except for the highest concentration (50 ng/mL) (Fig. 5A). Also, the results revealed that all concentrations of rhGal-9 were able to significantly augment IL-1βRAP expression compared to the control (Fig. 5B). Besides, there was a significant increase in IL-1βR expression in the co-treatment group versus the merely LPS-treated group. In contrast, no significant change was observed between the merely LPS-treated and the co-treatment group for expression of IL-1βRAP (Fig. 5C).

The effect of Gal-9 on NLRP3 expression

The results showed that rhGal-9 did not increase NLRP3 expression in any concentrations compared to the control (Fig. 6A). Moreover, NLRP3 expression was not changed significantly in the co-treatment group compared to the merely LPS-treated group (Fig. 6B).

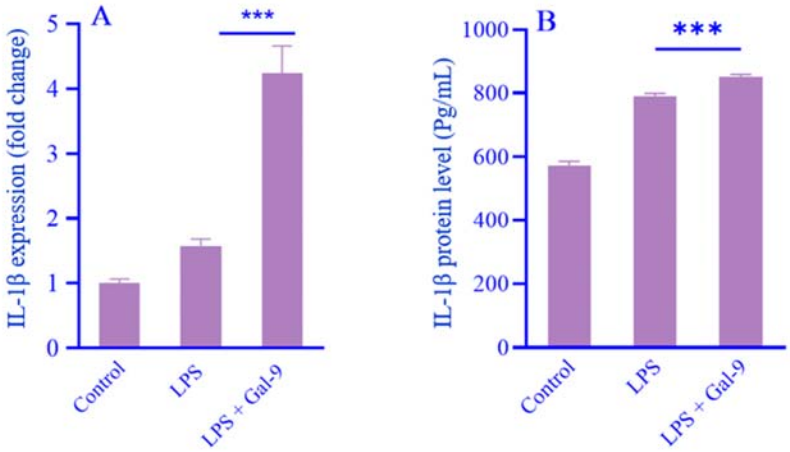

Fig. 4. The effect of Gal-9 in combination with LPS on (A) gene expression and (B) protein level of *IL-1β*. The specific concentration of rhGal-9 in combination with LPS was used for the treatment of U937 cells and following 24 h incubation the *IL-1β* gene and protein expression was measured by RT-PCR and ELISA methods, respectively. The data were achieved through two independent experiments, each performed in triplicate. Data are represented as the mean ± SD. ***$P < 0.001$ indicates significant differences between the defined groups. Gal-9, Galectin-9; LPS, lipopolysaccharide; IL, interleukin.

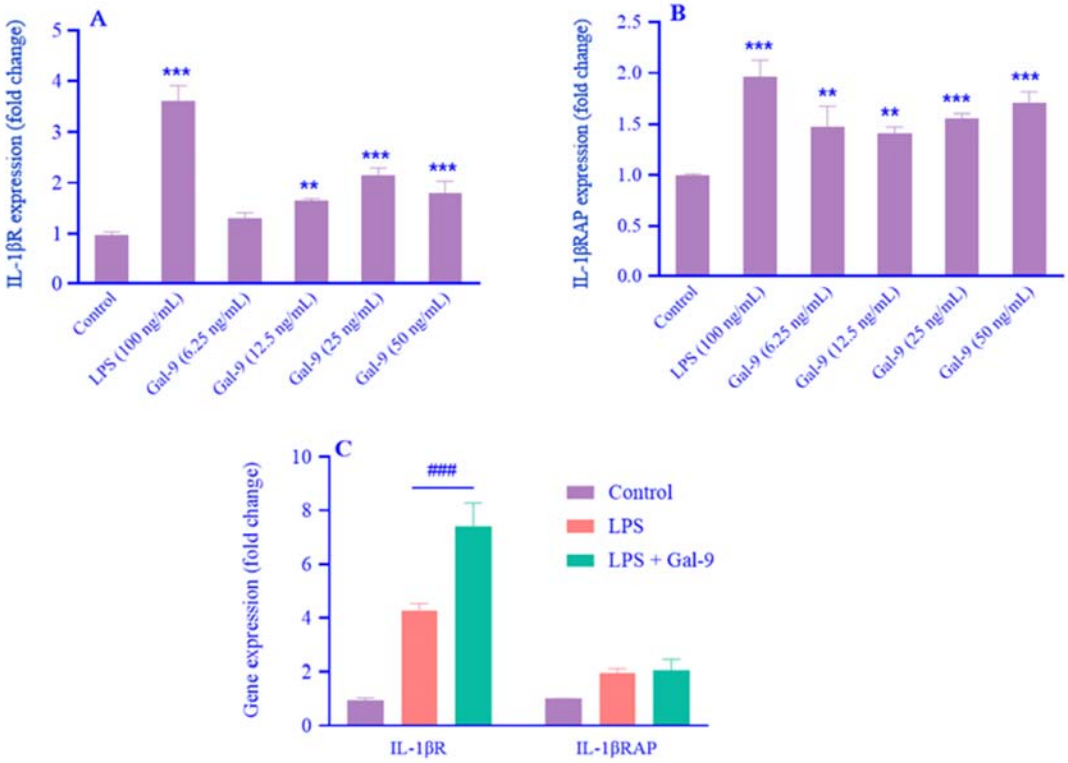

Fig. 5. The effect of Gal-9 alone or in combination with LPS on (A and C) *IL-1βR* and (B and C) *IL-1βRAP* gene expression. Different concentrations of rhGal-9 alone or in combination with LPS were added on U937 cells and after 24 h incubation, the *IL-1βR* and *IL-1βRAP* gene expression were studied by RT-PCR. The data were achieved through two independent experiments, each performed in triplicate. Data are represented as the mean ± SD. **$P < 0.01$ and ***$P < 0.001$ indicate significant differences compared to the control group; $^{###}P < 0.001$ between the defined groups. Gal-9, Galectin-9; LPS, lipopolysaccharide; IL, interleukin.

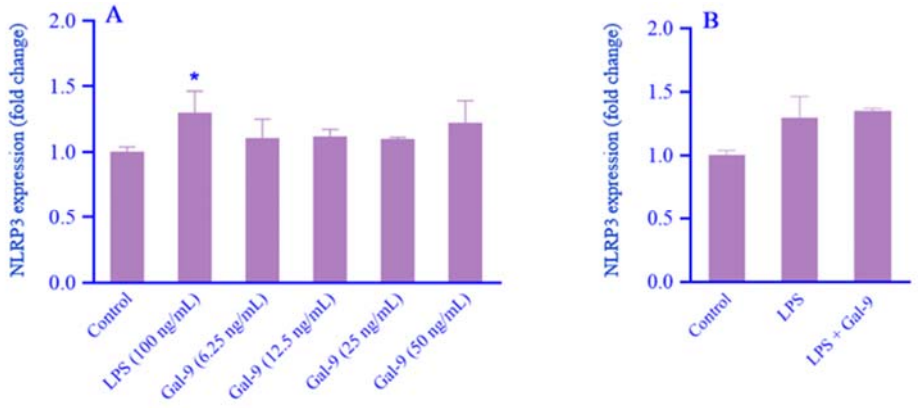

Fig. 6. The effect of Gal-9 (A) alone or (B) in combination with LPS on *NLRP3* gene expression. Different concentrations of rhGal-9 alone or in combination with LPS were added to U937 cells and after 24 h incubation, *NLRP3* gene expression was measured by RT-PCR. The data were achieved through two independent experiments, each performed in triplicate. Data are represented as the mean ± SD. *p < 0.05 indicates a significant difference compared to the control group. Gal-9, Galectin-9; LPS, lipopolysaccharide; NLRP3, NLR family pyrin domain containing 3.

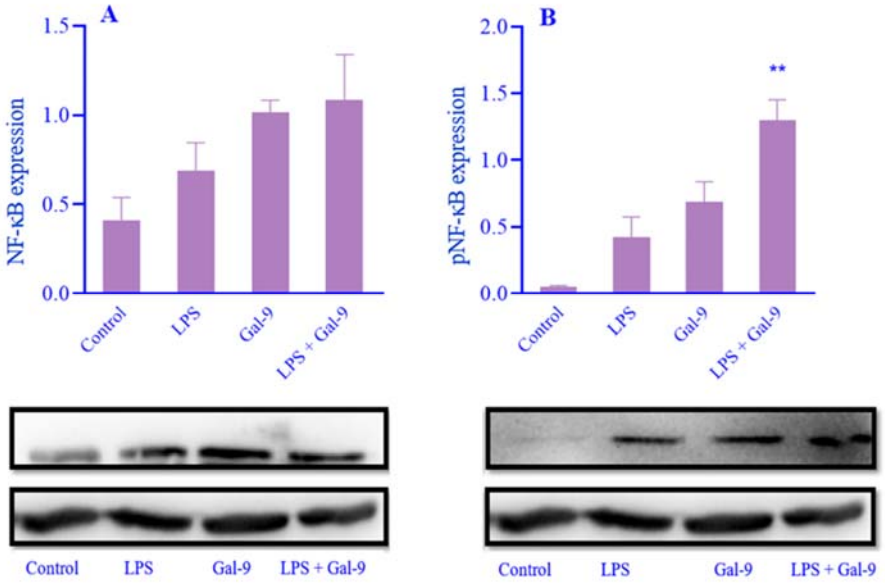

Fig. 7. The effect of Gal-9 alone or in combination with LPS on (A) NF-κB expression and (B) phosphorylation. rhGal-9 alone or in combination with LPS was added to U937 cells and after 24 h incubation, NF-κB expression and its phosphorylation were studied by western blotting, while β actin was considered for normalization (bands at the lower boxes). Data represent the median ± IQR of normalized protein expression with β-actin from experiments, which were performed in a triplicate manner. Statistical analysis was performed on protein expression using the Kruskal-Wallis, Dunn test, and Benjamini-Hochberg. **$P < 0.01$ indicates a significant difference in comparison with the control group. Gal-9, Galectin-9; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB.

The effect of Gal-9 on NF-κB phosphorylation

We found that rhGal-9 insignificantly increased the total NF-κB level compared to the control. Moreover, there was no significant difference between the co-treatment group and the merely LPS-treated group (Fig. 7A). Also, rhGal-9 provoked NF-κB phosphorylation compared to the control, although it was not statistically significant. In the co-treatment group, phosphorylation of NF-κB increased significantly in comparison with the untreated control but not with the merely LPS-treated group (Fig. 7B).

DISCUSSION

AML, the most common leukemia in adults, is assumed a fatal malignancy worldwide as opposed to human endeavor to discover an efficient therapy procedure. Primary AML cells have been reported to secrete high amounts of Gal-9, which forms an autocrine loop with its receptor (TIM-3) expressed on leukemic stem cells, leading to provoking multiple critical signaling pathways such as ß-catenin and NF-κB, which are influential in self-renewal and expansion of these cells (9,26). In the present study, we showed that Gal-9 can induce the proliferation of U937 cells in a gradual, steady trend up to a certain concentration, which is attributed to provoking ß-catenin and NF-κB pathways. However, the addition of further amounts of Gal-9 (over 100 ng/mL) neither increased the expansion rate of the U937 cells nor was toxic.

Also, we evaluated the effect of Gal-9 on *TIM-3* and *Gal-9* gene expression as crucial molecules in the TIM-3/Gal-9 autocrine loop. Similar to Kikushige *et al*. we found that Gal-9 cannot upregulate *TIM-3* expression (9).

Although Gal-9 did not affect the *TIM-3* gene expression, it significantly increased its own expression. Gal-9 binds to the IgV domain of TIM-3 and stimulates downstream signaling events (27,28). Previous studies have shown that in AML patients who did not respond to treatment, Gal-9 expression was elevated (29,30). Bojarska-Junak *et al*. have recently studied the prognostic role of Gal-9 and indicated that the upregulation of Gal-9 expression in B-cells of chronic lymphocytic leukemia patients correlates with the expression of two main proliferation markers, including Ki-67 and PCNA (10). According to the results of our study based on the synergistic role of Gal-9 in inducing its own expression and findings of other studies, targeting Gal-9 in the treatment of AML should be investigated in future studies. It was previously indicated that Gal-9 induces the mTOR signaling pathway, which affects Gal-9 expression and secretion (31,32).Therefore, Gal-9 induces its expression by promoting mTOR signaling. In the co-treatment groups, we observed that the expression of both TIM-3 and Gal-9 was lower than the merely LPS-treated group; of course, the reduction was only significant for Gal-9 expression. This might be associated with the potentially negative regulatory interplay between NF-κB and ß-catenin signaling pathways, which could be provoked following the treatment of the cells with both LPS and Gal-9 (33).

We also evaluated the effect of Gal-9 on IL-1β, which is assumed to be an indispensable inflammatory cytokine in the proliferation and therapy resistance of leukemia cells. We observed that Gal-9 can augment both IL-1β gene expression and its protein level in a concentration-dependent manner. During the time-dependent study, we found that the mRNA expression slightly increased, while the amount of protein experienced a steady reduction. mRNA translation may be suppressed in the presence of specific miRNAs or other regulatory mechanisms. Accordingly, Oladejo *et al.* have reported that Bta-miR-24-3p suppresses Gal-9 and decreases LPS-induced inflammatory response through TLR4/NF-κB signaling pathway (34). Anderson *et al*. have reported that Gal-9 induces tissue inflammation through binding to TIM-3, which is expressed on innate immune cells and increases TNF-α secretion as well (15). In another study, it was reported that both Gal-9 and agonistic antibodies against TIM-3 could increase TNF-α secretion in primary human AML cells (35).

In the current study, we found that in the co-treatment groups, IL-1β expression and protein levels were reinforced significantly, which could be due to the LPS and Gal-9 synergistic effect in provoking inflammation (36).

We also assessed the effect of Gal-9 on the expression of IL-1βR and its co-receptor (IL-1βRAP) and we found a significant increase in the expression of both molecules. Boer *et al*. have claimed that IL-1βRAP expression is upregulated on AML cells and, following stimulation by IL-1β, NF-κB, and P38-MAPK, signaling pathways are provoked, which results in the production of pro-inflammatory cytokines and chemokines and consequently, expansion and survival of AML cells (37).

Unlike most cytokines, IL-1β secretion is not through the direct classical secretory pathway and requires two signals, including activation of pattern recognition receptors and formation of the inflammasome complex (38). In this study, we also determined the impact of Gal-9 on NLRP3 expression, which showed no significant changes in NLRP3 expression.

Wang *et al*. have shown that Gal-9 decreases NLRP3 inflammasome activation by forming an autophagic cargo receptor and inducing protein degradation in primary peritoneal macrophages of C57BL/6J mice. Gal-9 knockout mice also showed increased NLRP3 inflammasome activation and NLRP3-related inflammation (39). Since we only evaluated the expression of the NLRP3 at the gene level, it seems that more studies on the evaluation of the effect of Gal-9 on NLRP3 are required in the future.

Finally, considering that NF-κB is a critical inflammatory signaling pathway and regulates the generation of several inflammatory elements such as cytokines and chemokines, we intended to investigate the effect of Gal-9 on NF-κB level and its phosphorylation. We found that treatment of the cells with both Gal-9 and Gal-9 + LPS could provoke NF-κB signaling and increase its phosphorylated form. Consistent with our findings, it was previously shown that Gal-9 could promote NF-κB signaling in AML leukemic stem cells through binding to TIM-3 (9). The involvement of the Wnt pathway in AML pathogenesis has been indicated in many studies (40-43). It is worth mentioning that IL-1β regulates NF-κB through two independent pathways by releasing NF-κB from its inhibitory proteins (44,45). Moreover, it has been indicated that NF-κB can augment Wnt-signaling, leading to the expansion of tumor-initiating cells (46). Kaler *et al*. have shown that IL-1β induces canonical Wnt signaling pathways and promotes cancer cell proliferation (47). Therefore, the increase of IL-1 β in the presence of Gal-9 probably participates in the activation of the NF-κB pathway. However, more mechanistic studies are required to identify the relationship between Gal-9, IL-1 β, and the NF-κB pathway.

CONCLUSION

We found that Gal-9 may induce inflammation through NF-κB signaling and augmentation of IL-1β production. Meanwhile, due to the importance of IL-1β in the proliferation, therapy resistance, and exacerbation of AML, targeting both Gal-9 and IL-1β as key molecules and interfering with the potential cross-talk between them could influence AML therapy. However, more mechanistic studies are required to confirm the crosstalk between the TIM-3/Gal-9 pathway and IL-1β generation in AML pathogenesis.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

R. Falak and F. Nasri conceived the current idea. N. Behranvand set up the research experiments. N. Behranvand and F. Nasri performed the main experiments and wrote the initial manuscript. N. Esmaeil wrote some parts of the manuscript and revised it. N. Esmaeil, M. Vakilymoghaddam, and M. Davoodzadeh performed some parts of the experiments. E. Safari and R. Falak revised the final manuscript. All authors read and approved the finalized manuscript.

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