Cytokine profile and nitric oxide levels in peritoneal macrophages of BALB/c mice exposed to Fucose-Mannose Ligand of Leishmania infantum combined with Glycyrrhizin

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Abstract

Background The Fucose-Mannose Ligand (FML) of Leishmania infantum is a complex glycoprotein which does not elicit adequate immunogenicity in human. In recent years, adjuvant compounds derived from plants have been used for improving the immunogenicity of the vaccines. Glycyrrhizin (GL) is a natural triterpenoid saponin that has known immunomodulatory activities. In the present study, we investigated the effects of a co-treatment with FML and GL on the production of cytokines and nitric oxide (NO) by macrophages, in vitro . Methods Lipopolysaccharide (LPS) stimulated murine peritoneal macrophages were treated with FML (5 µg/ml) of Leishmania infantum and various concentrations of GL (1 µg/ml, or 10 µg/ml or 20 µg/ml). After 48h of treatment, cell culture supernatants were recovered and the levels of TNF-α, IL-10, IL-12p70, and IP-10 were measured by sandwich ELISA and NO concentration by Griess reaction. Results Our results indicated that the treatment of activated macrophages with FML plus GL leads to enhanced production of NO, TNF-α, IL-12p70, and reduction of IL-10 levels in comparison with FML treatment alone. Conclusions We, therefore, concluded that GL can improve the immunostimulatory effect of FML on macrophages and leads to polarization of them toward an M1-like phenotype.

1. Background

Visceral leishmaniasis (VL) or kala-azar, a cosmopolitan vector-borne zoonotic disease, is the most severe and fatal form of leishmaniasis if not diagnosed and untreated in time. The disease is caused by parasitic protozoan species of the *Leishmania donovani* complex and is transmitted by sandflies (1). It is one the most important parasitic diseases beside many parasitic diseases in Iran (2-5).

In the recent past, the parasites have developed resistance to the existing drugs and also due to lack of effective human vaccine against VL, lead to increase in the incidence of VL (6-9). Different generations of vaccines based on different antigens of the parasite have been examined to fulfill the hopes for an appropriate concept for leishmaniasis treatment, and prevention or induce protection with long-term immunity (9-12).

Among the various antigens that serve as targets for VL vaccine design, fucose-mannose ligand (FML) has attracted much attention owing to its excellent immunoprotective properties against experimental VL in several animal models (13). The FML is a glycoprotein antigen which is present both in amastigotes and in motile promastigotes of *L. donovani* complex. Even though the FML is a potent immunogen in rabbits and dogs (e.g., Leishmune® a vaccine for canine VL consists of FML and saponin) (14), it does not have adequate immunogenicity in human (9, 15).

Previous studies have demonstrated that macrophages play a pivotal role in the outcome of *Leishmania* infection depending on type of macrophages; classically activated (M1) macrophages as efficient type against *Leishmania* parasites or alternatively activated (M2) macrophages as favoring survival and growth of *Leishmania* parasites (16, 17). In response to different microbial stimuli and immune status of

the microenvironment, naive macrophages (M0) differentiate to either M1 or M2 subpopulation with different patterns of cytokine production and distinct properties. Concerning stimuli that can affect shift macrophages toward M1 or M2 subpopulation, in a recent study we evaluated the immunomodulatory effects of FML on macrophages (18). Our findings showed that although the FML significantly increases nitric oxide (NO), IL-12p70 and IP-10 production in macrophages, but cannot alter TNF-α production in them (18). The most surprising aspect of this study was that FML significantly increases the production of IL-10, an immunosuppressive cytokine, from macrophages (18).

Glycyrrhizin (GL) is a natural triterpenoid saponin derived from the root of licorice (*Glycyrrhiza glabra*) that has been associated with numerous pharmacologic effects, including anti-bacterial, anti-inflammatory, anti-oxidant, anti-viral, anti-tumor, hepatoprotective, and immunomodulatory activities (19-22). Stimulation of IL-12 and NO production and suppression of IL-10 production from macrophages (23, 24), augmentation of NK cell activity (23), up regulation of costimulatory molecules on dendritic cells (19), increasing of T cells proliferation, reduction of IL-4 production from T cells, direction of immune response toward Th1 (19), are the most important known immunomodulatory activities of GL.

It is well known that the GL plays an important role in immunomodulatory activities such as stimulation of IL-12 and NO production and suppression of IL-10 production from macrophages (23, 24). The GL also plays a central role in the augmentation of NK cell activity, up-regulation of costimulatory molecules on dendritic cells, increasing of T cells proliferation, reduction of IL-4 production from T cells, and direction of the immune response toward Th1, (19).

With regard to new improvements in produce human VL vaccine by using purified FML and considering the importance of macrophages in protection and control against VL, it seems that characterization of the immunomodulatory effects a combination of FML/GL on macrophages can be useful for finding ways to increase the immunogenicity of FML and vaccine development. Therefore, for the first time, we investigated the effects of FML/GL on production of cytokines and NO by macrophages, *in vitro*.

With regard to the improvements in producing the human VL vaccines by using purified FML and considering the importance of macrophages in protection and control of VL, it is important to characterize the immunomodulatory effects of combination of FML/GL on macrophages which can be useful in finding ways to increase the immunogenicity of FML and vaccine development.

2. Methods

2.1. Animals

For the experimental studies, a total of seven 6-8 week-old female BALB/c mice were used from Razi Institute (Mashhad, Iran). The mice were fed standard mouse chow ad libitum throughout the study and maintained under the standard conditions according to the protocol. The experimental protocols used in the study were approved by the Animal Ethics Committee of North Khorasan University of Medical Sciences, Bojnurd, Iran (95-914/p).

2.2. Leishmania promastigote culture and FML extraction

L. infantum promastigotes (MCAN/IR/07/Moheb-gh) were grown at 26 °C in brain heart infusion broth (37 g/l; Himedia, India) supplemented with 10% of fetal bovine serum (Gibco, UK), hemin (0.01 g/l) and folic acid (0.02 g/l; Sigma, MO, USA).

The stationary phase growth medium was centrifuged at 6000xg for 10 min to collect the promastigotes. The pellet containing promastigotes was washed with cold phosphate-buffered saline (PBS) and was stored at -20 °C until further analysis. Aqueous extraction of FML was carried out as explained earlier by Foroughi-Parvar et al. (25). Briefly, frozen pellets of the parasite were mixed with cold distilled water and centrifuged at 6000xg for 10 min to collect the supernatant. This step was repeated once again, and both the supernatants were combined and boiled for 15 min at 100 °C. The sample was then centrifuged, and the supernatant was lyophilized and subjected to chromatography by loading 2 ml of lyophilized sample in cold deionized distilled water (10 mg/ml) on 100 × 1.6 cm column of P10 Bio-Gel (Biorad, UK) to purify the FML. The collected FML samples were analyzed further for the carbohydrate content and the presence of 10–96 kDa bands corresponding to FML glycoprotein on 10% SDS-PAGE. The purified FML samples were lyophilized and stored at -20 °C for further use.

2.3. Isolation peritoneal macrophages

Seven female BALB/c mice were sacrificed by CO_2 euthanasia. Isolation of peritoneal macrophages from them were performed based on the procedure described by Bibak *et al* (26). Briefly, murine peritoneal cells were harvested by lavage of the peritoneal cavity with 10 ml of RPMI 1640 medium (Invitrogen, Germany). The cells were centrifuged at 200 × g for 10 min and washed them in PBS/cold ddH_2O. The cells were then cultured in RPMI 1640 medium in petri dishes at 37 °C for 4 h. Petri dishes were carefully washed using Hanks' solution to remove the non-adherent cells. The cells adhered to the petri dishes were trypsinized and the concentration of the cells were adjusted to 1×10^6 cells/ml in the RPMI medium (RPMI medium with 10% FCS (Invitrogen, Germany) containing 50 IU/ml penicillin/streptomycin (Sigma-Aldrich, USA).

2.4. Treatment of peritoneal macrophages with a combination of FML and GL

Isolated peritoneal macrophages stimulated with 10 µg/ml of LPS at 37 °C and 5% CO2 for 4 hr. The activated macrophages treated with FML together with varying concentrations of GL to assess the immunomodulatory effects of combination of FML and GL. To prepare activated macrophages, 2×10^5 macrophage cell suspensions (200 µl/well on 96-well flat-bottom plates), in each well, was treated with 10 µg/ml of LPS from Escherichia coli O111: B4 (Sigma, USA) in a complete RPMI medium. Thereafter, 5 µg/ml of FML and 1, 10, or 20 µg/ml of GL were added to activate cells/well in triplicate as described in the earlier studies (25, 27). The cells were cultured at 37 °C and 5% CO2 for 48hr. In control group or PBS group, activated macrophages were cultured in the presence of PBS alone at the same volume as the other additions (10µl/ml). For NO assay, we used only complete RPMI-1640 medium as the blank control group. After 48h, culture supernatants from each well of 96-well plate were collected and stored at -80 °C

until further analysis. Each experiment was performed in triplicate. The different study groups are shown in Table 1. MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (Merck, Germany) assay was used to evaluate the macrophage viability after 48hr incubation at 37 °C with different concentrations of GL (0.1, 1, 10, 20, 50 and 100 µg/ml).

Table 1: Treatment of peritoneal macrophages with FML and different concentrations of GL in presence of LPS.

No. Study group	LPS (10 µg/ml)	FML (5 µg/ml)	Combination of FML (5 µg/ml) and GL (1 µg/ml)	Combination of FML (5 µg/ml) and GL (10 µg/ml)	Combination of FML (5 µg/ml) and GL (20 µg/ml)	*PBS
1	+	-	-	-	-	+
2	+	+	-	-	-	-
3	+	-	+	-	-	-
4	+	-	-	+	-	-
5	+	-	-	-	+	-

* Phosphate buffered saline.

2.5. Nitric oxide and cytokine assay

The culture supernatants were evaluated for the stable end-products of NO, nitrates, and nitrites, using the Standard Griess Reagent according to the Caymanchem instruction manual (Cayman Chemical, USA). Levels of NO in different treatment groups were determined by reading the absorbance at 540 nm in a microplate reader (BioTek). The mean optical density (OD) values of the blank were subtracted from the mean OD values of the test. Using the standard curve obtained with serial dilutions of sodium nitrite as the standard, the concentration of the nitrite was calculated. TNF-α, IL-10 and IL-12p70 were measured in the cell culture supernatants using sandwich ELISA kits according to the instructions of the manufacturer (eBioscience, USA). The minimum detectable concentration was 5 pg/ml for both IL-12p70 and IL-10, and 1 pg/ml for TNF-α.

2.6. Statistical analysis

GraphPad Prism software version 5.0 (GraphPad Software, USA) was used for statistical analysis of the data. Data distribution was analyzed by Kolmogorov–Smirnov test. According to the results of the normality test, one-way ANOVA followed by Dunns or Tukey post-test or non-parametric Kruskal–Wallis test were used for statistical comparisons. Data are shown as the mean ± SD of three independent experiments. P values < 0.05 were considered statistically significant.

3. Results

After the treatment of LPS stimulated macrophages with FML and different concentrations of GL, we determined the viability of cells with MTT reduction assay, measured the levels of TNF-α, IL-10 and IL-12p70 using sandwich ELISA method and NO concentration using Griess reaction.

Results of the MTT assay indicated that FML at 5μg/ml co-treatment with GL at 1, 10, and 20 μg/ml had not cytotoxic effect on activated macrophages, thus these concentrations were used for NO and cytokine assay. Since the activated macrophages treated with GL at 50 and 100 μg/ml exhibited low viability (<90%) and hence were not included for further analysis (Fig. 1).

The concentrations of NO in supernatants of activated the macrophages treated with FML were significantly higher than the activated macrophages treated with PBS (11.20 ± 1.80 μM/ml vs. 7.84 ± 1.54 μM/ml, $^{*}P$=0.013). As shown in Fig. 2, treatment of activated macrophages with FML (5 μg/ml) plus GL (at concentration of 10 and 20 μg/ml) significantly increased NO production in comparison with FML treatment alone (11.20 ± 1.80 μM/ml vs. 14.40 ± 1.90 μM/ml, $^{*}P$= 0.026 and 15.60 ± 1.66 μM/ml, $^{**}P$= 0.003) (Fig.2).

After measurement of TNF-α in the culture supernatant of activated macrophages, no significant differences were found between FML-treated macrophages and PBS treated macrophages (577.6±73.4 pg/ml vs. 526±45.6 pg/ml, P= 0.223). However, the results of our study that treated with FML in combination with 20 μg/ml GL significantly increase the production of TNF-α in activated macrophages compared to activated macrophages treated with FML alone (782.0 ± 69.49 vs. 577.6±73.4 pg / ml, $^{**}P$=0.0019) (Fig. 3).

The FML significantly increased IL-10 production from activated macrophages compared to the activated macrophages treated with PBS (1601 ± 54.11 pg/ml vs. 1242 ± 79.68 pg/ml, $^{**}P$=0.005). The concentration of IL-10 in the supernatant of activated macrophages treated with combination of FML and GL (at concentrations of 10 and 20 μg/ml) were significantly lower than the concentration of IL-10 in the supernatant of only FML-treated activated macrophages (1601 ± 54.11 pg/ml vs. 1028±46.2 pg/ml, $^{***}P$<0.001, and 722.2±147.8 pg/ml, $^{***}P$<0.001)

Our results showed that the co-treatment with FML and GL (at concentrations of 10 and 20 μg/ml) significantly increase the production of IL-12p70 from activated macrophages compared with activated macrophages treated with FML alone (749.3 ± 47.5 pg/ml vs. 991.6± 79.1 pg/ml, $^{**}P$ < 0.001, and 964.6± 83 pg/ml, $^{***}P$ < 0.0004) (Fig.5). Also, there was a significant difference between concentration of IL-12p70 produced from activated macrophages treated with FML and the activated macrophages treated with PBS (749.3 ± 47.5 pg/ml vs. 514.6 ± 48.37 $^{**}P$=0.002) (Fig.5).

4. Discussion

Previous studies have shown that FML does not provide adequate immunogenicity and cannot efficiently stimulate immune response of the macrophages (9, 15). GL is a well-known immunomodulatory

component that stimulates immune response of the infected macrophages (28). Therefore, we assumed that treatment with a combination of FML and GL can improve the efficiency of macrophages against VL infection through the induction of protective cytokines and reactive nitrogen species (RNS). To evaluate this hypothesis, we studied the effects of FML in combination with GL on the production of TNF-α, IL-12p70, IL-10 and NO in the murine peritoneal activated macrophages *in vitro*.

Our results indicated that the treatment of activated macrophages with FML plus GL leads to enhanced production of NO, TNF-α, IL-12p70 in comparison with FML treatment alone. Surprisingly, we found that co-treatment of macrophages with FML and GL markedly inhibits the production of IL-10 compared to FML treatment alone. Cytokine profile and NO levels in macrophages treated with a combination of FML and GL were similar to the patterns described in earlier studies about M1 macrophages (16).

NO has been demonstrated to be a principal effector molecule responsible for mediating intracellular killing of *Leishmania* parasites, *particularly L. donovani* complex (29). The current study has shown that GL helps in enhancing the production of NO from FML-treated activated macrophages (Fig.2). In accordance with the present results, previous studies have demonstrated that FML or GL alone enable to increase NO production from activated macrophages (19, 24).

The TNF-α plays a critical role in the control of intracellular pathogens, especially those that infect macrophages (30). It has been demonstrated that TNF-α is required for the control of VL infection in human through stimulation of IFN-γ production (30). Similarly, Tumang *et al.* also have shown that endogenous TNF-alpha appears to be critical for both the initial acquisition of resistance to *L. donovani* and resolution of experimental VL infection (31). We know from our previous study that FML could not enhance TNF-α levels in the activated macrophages (19). In the current study, we found that the treatment of activated macrophages with FML plus GL increases the production of TNF-α in them. This outcome is contrary to that of Liu *et al*. and Fu *et al*. who found that GL inhibits the secretion of TNF-α by LPS-stimulated macrophages and mammary epithelial cells, respectively (32, 33). It is difficult to explain these discrepancies, but it may be due to inhibition of LPS-induced nuclear factor-κB by GL without suppression of nuclear factor-κB induced by MyD88-dependent downstream signaling (33).

VL is characterized by the absence of cytokines such as IFN-γ and IL-12 and cure of VL is associated with a restoration of these cytokines (34). Several reports have shown that IL-10 and IL-12 play opposite roles in control of VL infections (35). The IL-10 is associated with progression VL and IL-12 with the control of disease caused by *Leishmania donovani* complex (35). In a recent study, we demonstrated that FML increases the levels of both IL-12p70 and IL-10 in activated macrophages (19). In the present study, we have shown that use of FML in combination with GL exerts strong booster effect on IL-12 and suppressive effect on IL-10 production in activated macrophages. This finding is consistent with that of Dai *et al.* who demonstrated the ability of GL to enhance LPS-induced IL-12 production by peritoneal macrophages (23). The results of the present study are also consistent with our earlier observations, which showed that GL significantly inhibits IL-10 secretion by LPS-stimulated RAW264.7 cells, a mouse macrophage cell line (32).

5. Conclusion

Taken together, our findings suggest that GL can improve immunostimulatory effect of FML on macrophages and leads to their polarization toward an M1-like phenotype, which is an efficient phenotype against *Leishmania* parasites. More studies using animal models of VL are needed to evaluate the protective effect of the combination of FML/GL.

List Of Abbreviations

Visceral leishmaniasis (VL), Fucose-Mannose Ligand (FML), Lipopolysaccharide (LPS), Nitric Oxide (NO), Glycyrrhizin (GL), Phosphate buffered saline (PBS), Microwave Theory and Techniques (MTT), Optical Density (OD)

Declarations

Ethics approval and consent to participate

The experimental protocols that have been used in this research was approved by the Ethical Committee of the North Khorasan University of Medical Sciences, Bojnurd, Iran (IR.NKUMS. REC.1395.06).

Consent for publication

Not applicable

Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Competing interests

Not applicable

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Authors' contributions

Dr. Namdar and Dr. Shafiei who have performed the experiments and writing, Dr. Hatam who was prepared the FML Ag, Dr. Zolfaghari Emameh and Dr. Aspatwar who helped in the scientific design, writing and revising of the manuscript.

Disclosure statement

The authors reported no potential conflict of interest relevant to this article.

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Figures

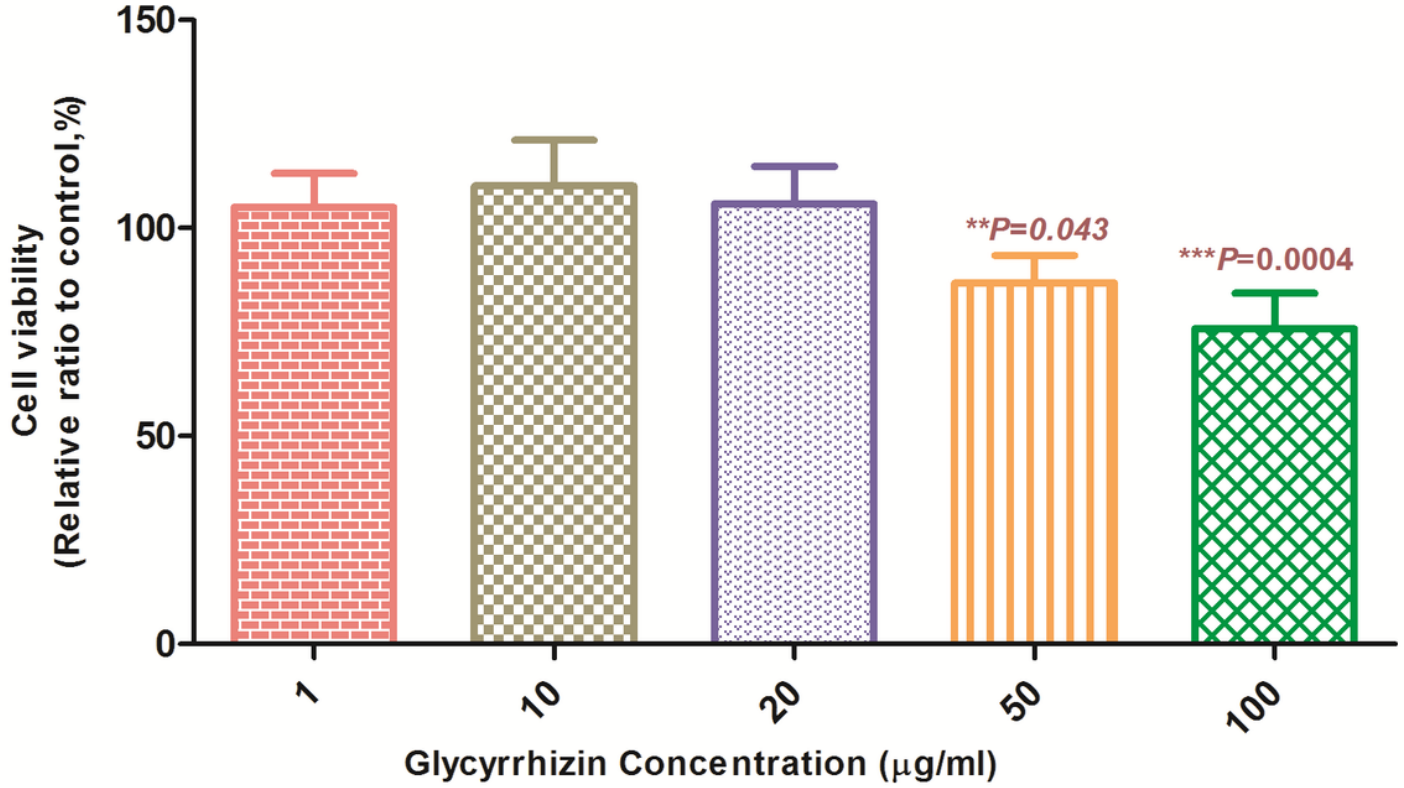

Figure 1

Peritoneal macrophage viability in the presence of different concentrations of Glycyrrhizin (GL). The average values of MTT reduction in activated macrophages treated with different concentrations of GL were the same in all the groups except at concentrations of 50 and 100 μg/ml (P > 0.05).

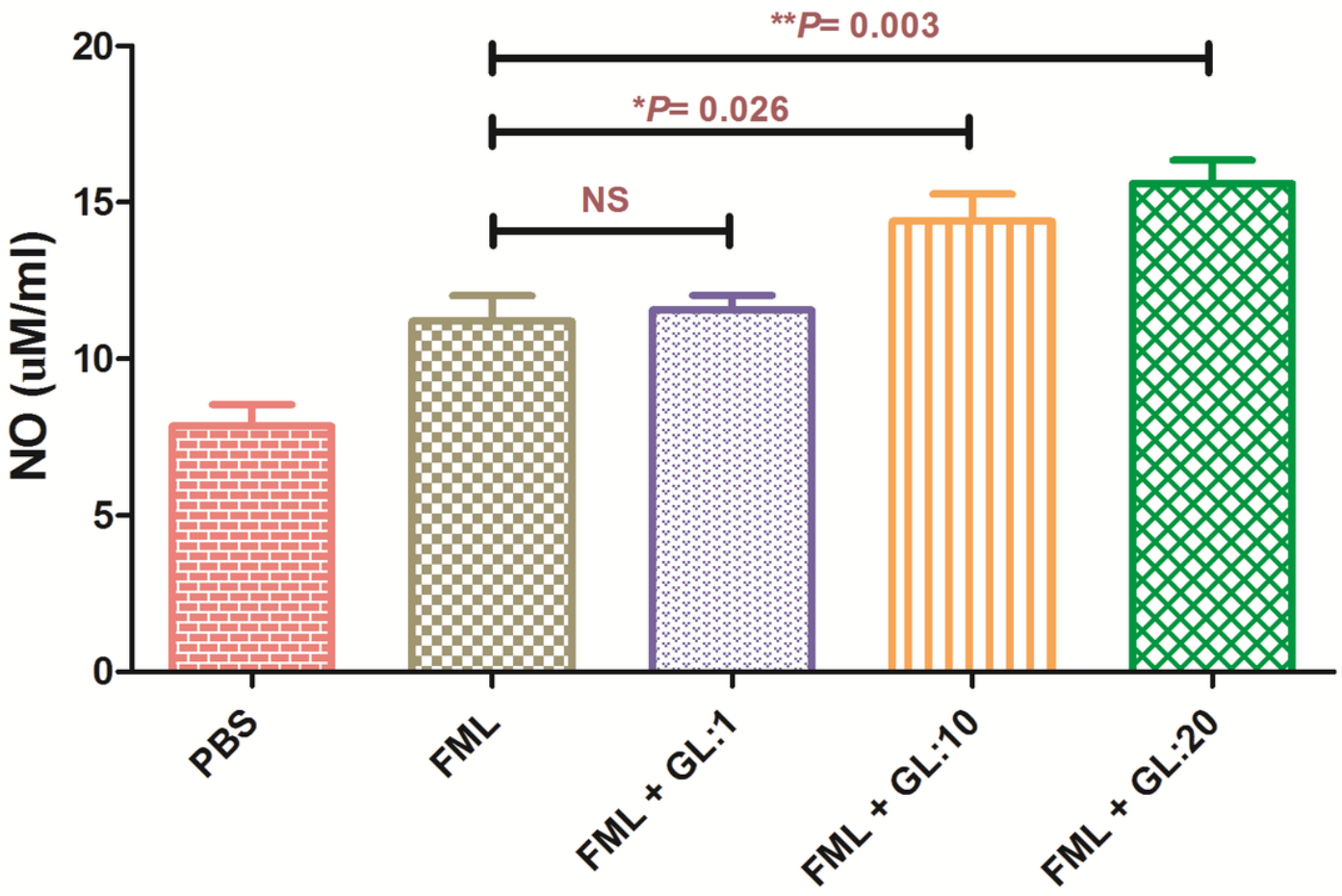

Figure 2

The effect of the combination of Fucose-Mannose Ligand (FML) with Glycyrrhizin (GL) on the production of Nitric Oxide (NO) by activated macrophages. Co-treatment with FML and GL significantly increased NO production in activated macrophages in comparison with activated macrophages treated with FML alone or PBS. Results represent mean (mean ±SD) of three independent experiments with macrophages from seven mice per experiment (*P < 0.05, **P < 0.01, and ***P < 0.001). PBS; Phosphate-buffered saline.

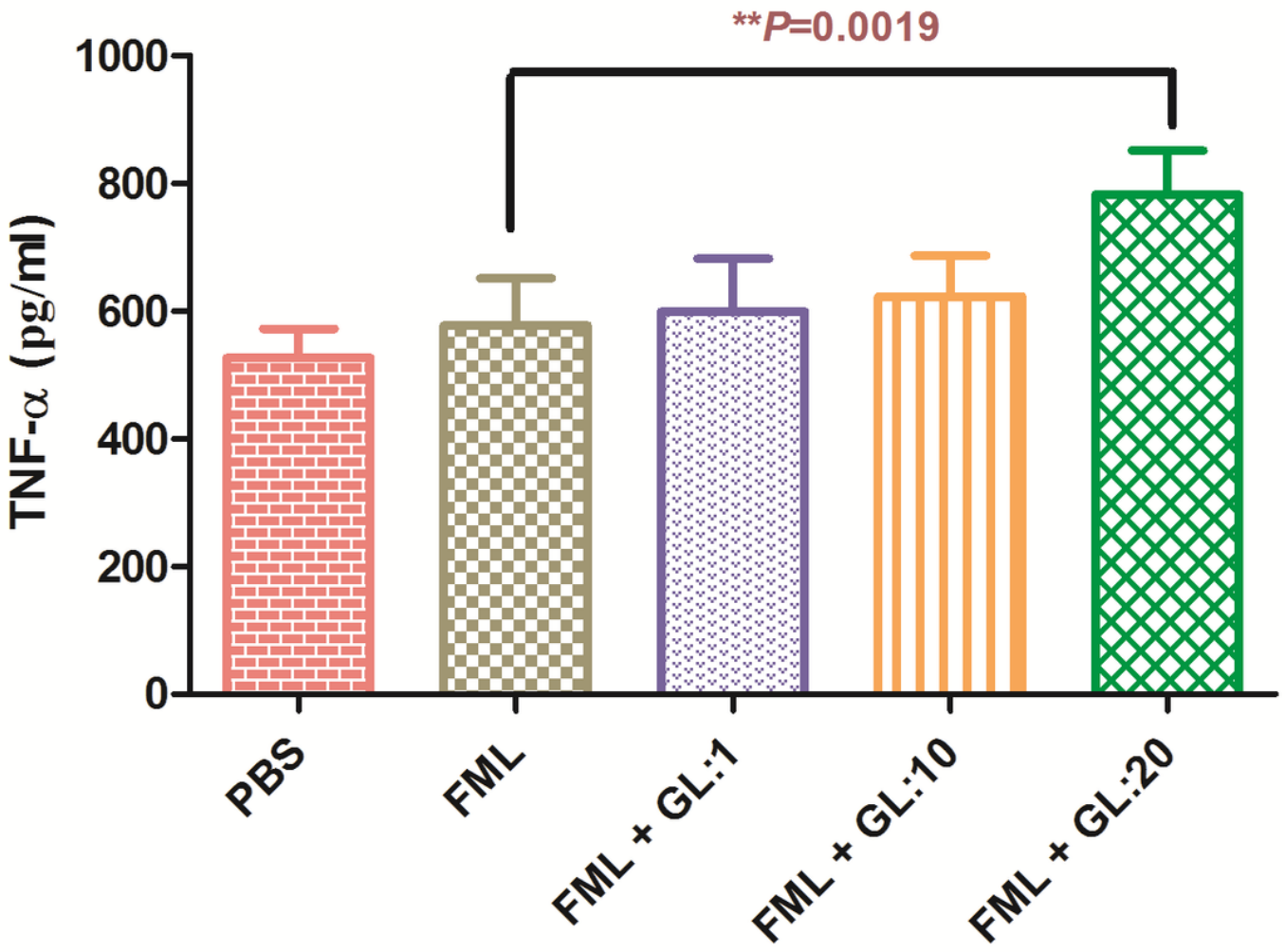

Figure 3

The effect of the combination of Fucose-Mannose Ligand (FML) with Glycyrrhizin (GL) on the production of TNF-α by activated macrophages. The production of TNF-α was significantly higher in the macrophage treated with the combination of FML and GL at high concentration (20 μg/ml) compared to the activated macrophages treated with FML alone (**P=0.0019). Results represent mean (Mean ± SD) of the three independent experiments with macrophages from seven mice per experiment (*P < 0.05, **P < 0.01, and ***P < 0.001). PBS; Phosphate-buffered saline.

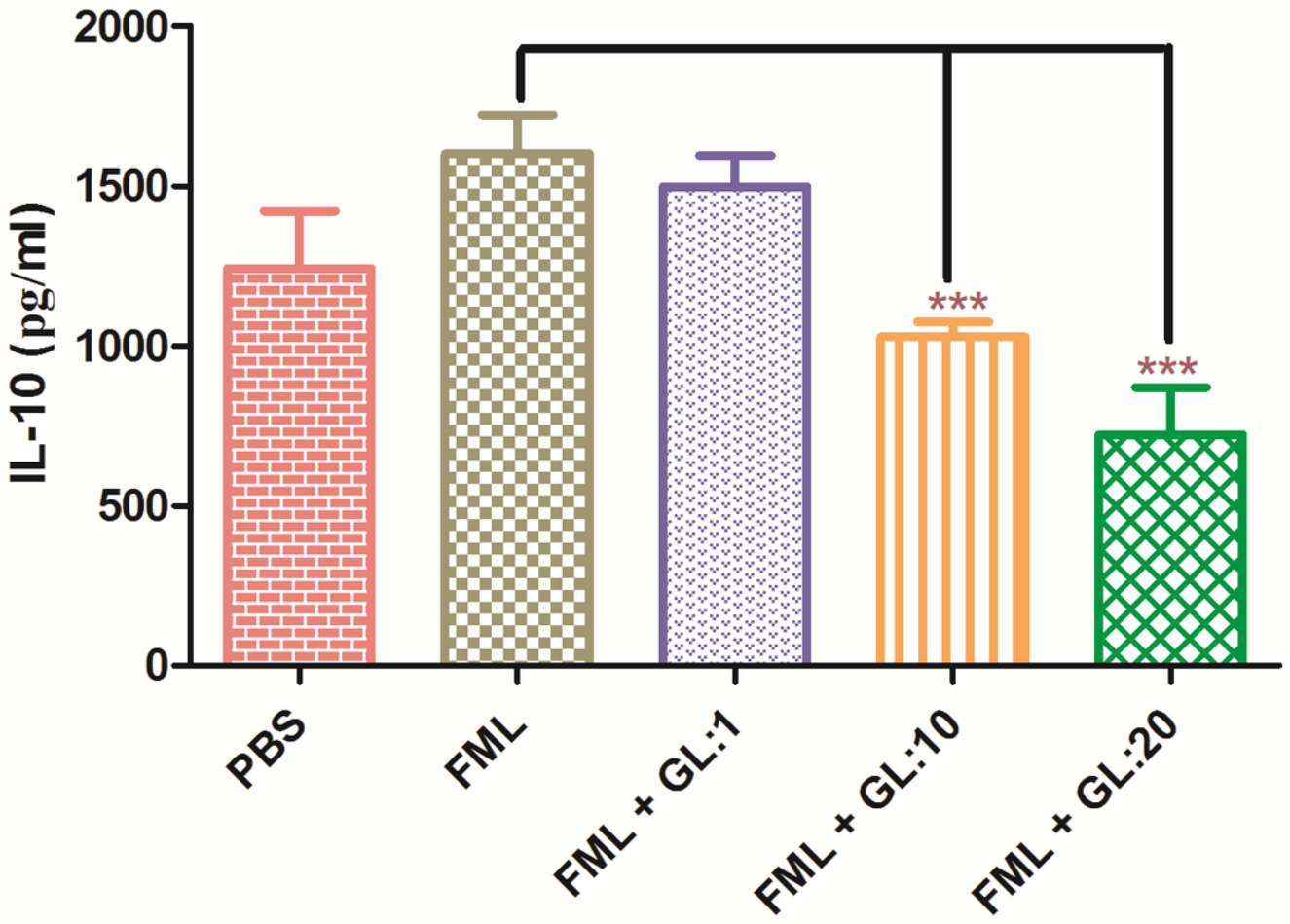

Figure 4

The effect of the combination of Fucose-Mannose Ligand (FML) with Glycyrrhizin (GL) on the production of IL-10 by activated macrophages. Treatment with FML together with GL at 10 and 20 µg/ml concentrations significantly reduced IL-10 production by activated macrophages compared to the FML treatment alone. Results are expressed as mean ± SD of triplicate cultures. Results represent mean (mean ± SD) of three independent experiments with macrophages from seven mice per experiment (*P < 0.05, **P < 0.01, and ***P < 0.001). PBS; Phosphate-buffered saline.

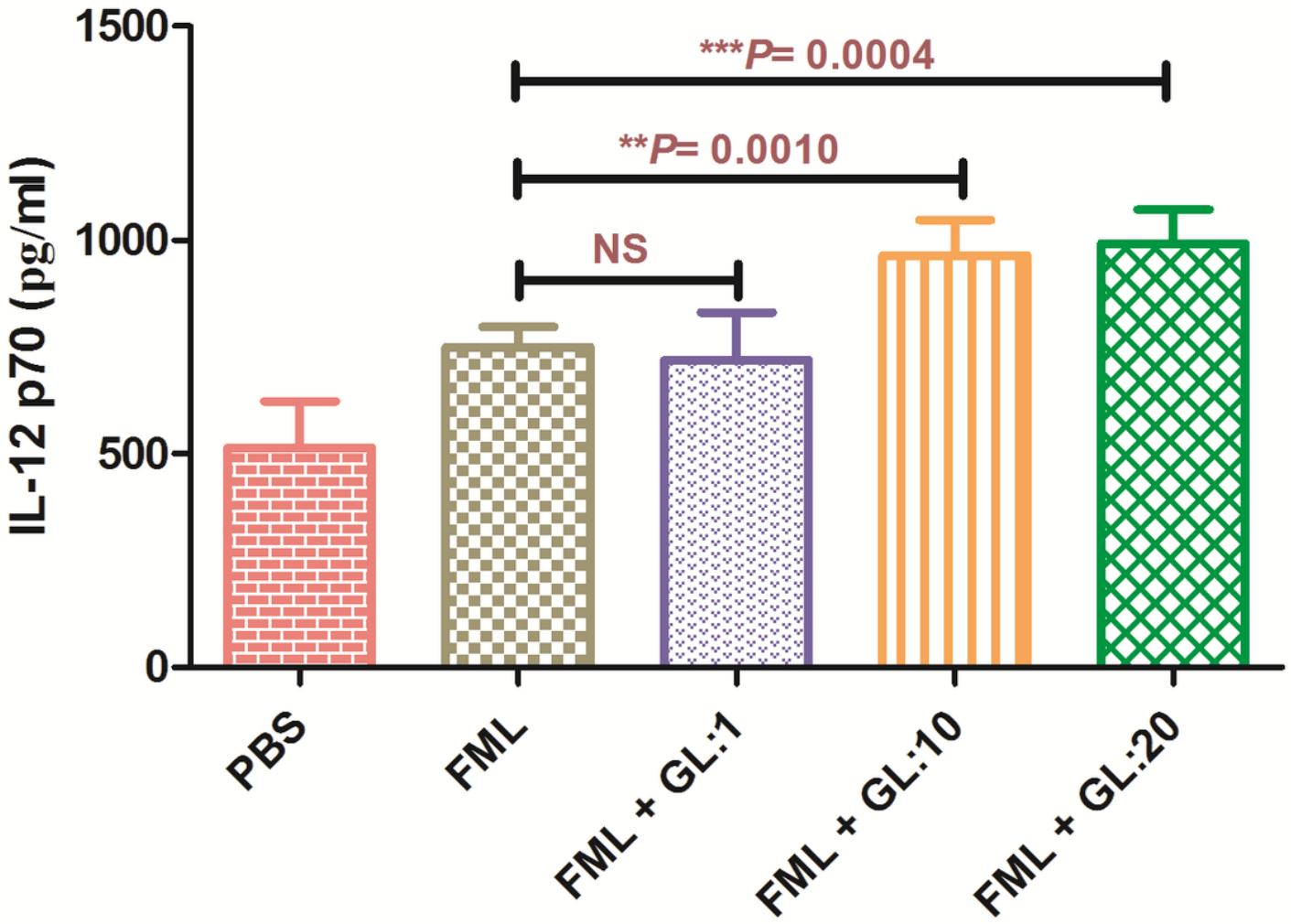

Figure 5

The effect of combination of Fucose-Mannose Ligand (FML) with Glycyrrhizin (GL) on the production of IL-12p70 by macrophages. The FML with 10 and 20 µg/ml concentrations of GL significantly increased IL-12p70 levels in activated macrophages. Results are expressed as mean ± SD of measurements from triplicate cultures. (*P < 0.05, **P < 0.01, and ***P < 0.001). PBS; Phosphate-buffered saline.

Supplementary Files

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- Graphicabstract.jpg