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Echinococcus granulosus' laminated layer immunomodulates nitric oxide, cytokines, and MMPs in PBMC from rheumatoid arthritis patients

F. Ameur[1], M. Amri[1], S. Djebbara[1], I. Soufli[1], R.-S. Boussa[1], S. Benazzouz[1], I.-M. Boutemine[1], S. Benkhelifa[1], M. Bouchemal[1], C. Mekhloufi-Dahou[2], F. Hanni[3], M. Yakoubi[4], S.T. Lefkir[5], S. Abdellaoui[5], A. Arroul-Lammali[1], N.S. Idris[6], H. Belguendouz[1] and C. Touil-Boukoffa[1,7]

[1]Team 'Cytokines and NO Synthases: Immunity and Pathogeny', Laboratory of Cellular and Molecular Biology, Faculty of Biological Sciences, University of Science and Technology Houari Boumediene, Algiers, Algeria; [2]Rheumatology department, Bab El Oued Hospital, Algiers, Algeria; [3]Rheumatology department, Ben Aknoun Hospital, Algiers, Algeria; [4]Orthopedic department, Ben Aknoun Hospital, Algiers, Algeria; [5]Rheumatology department, Beni Messous Hospital, Algiers, Algeria; [6]Surgery department, Djillali Belkhenchir Hospital, Algiers, Algeria and [7]Algerian Academy of Sciences and Technologies, Algiers, Algeria

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects the joints. Treatments are symptomatic and can induce side effects in some patients. In this sense and based on previous studies, our aim was to investigate the ex vivo immunoregulatory effect of the laminated layer (LL) during rheumatoid arthritis. LL is the outside layer of parasitic cyst of the helminth *Echinococcus granulosus.*

Our main objective was to study the effect of LL on nitric oxide (NO) and cytokines production, matrix metalloproteinases (MMPs) activities, inducible NO synthase (iNOS) and nuclear factor κappa B (NF-κB) expression. In this context, cultures of peripheral blood mononuclear cells (PBMC) from Algerian RA patients in active (ARA) and inactive (IRA) stage of the disease were stimulated with LL extract (50, 100, 150μg/mL). However, PBMC from ARA patients were stimulated with methotrexate (MTX; 0.5μg/mL) and biological disease modifying anti-rheumatic drugs (bDMARDs): anti-TNFα (10μg/mL), anti-IL6 (10μg/mL), anti-CD20 (10μg/mL), alone or combined with LL (50μg/mL).

Our results showed that LL reduced NO, TNF-α, and IL-17A production, MMP9/2 activities, and iNOS/NF-κB expression in PBMC from ARA patients. Concomitantly, LL increases IL-10 and TGF-β1 production in the same cultures. Interestingly, the decrease in NO production induced by bDMARDs was greater in association with LL.

Collectively, our findings indicate a strong immunoregulatory effect of LL on NO, MMPs, and cytokines. LL probably acts through the NF-κB pathway. The development of biodrugs derived from LL of *E. granulosus* could be a potential candidate to modulate inflammation during RA.

Introduction

It has been known for many years that the incidence of auto-inflammatory diseases has increased in developed countries (Bach 2019). In this sense, it has been suggested that the reduced exposure to microorganisms resulting from improved sanitary conditions promotes the development of immune-mediated diseases. This theory has been called the hygiene hypothesis or, more recently, the old friends' hypothesis (Murdaca *et al.* 2021; Pfefferle *et al.* 2021). Helminths represent the major groups of organisms implicated in this spike of auto-inflammatory disease incidence (Harnett and Harnett 2017). Indeed, infection with the helminths or treatment with their secretory proteins demonstrated a protective effect against immune-mediated diseases in animals and humans (Hernandez *et al.* 2013; Soufli *et al.* 2015).

Echinococcus granulosus is a helminth that causes cystic hydatid disease in humans. Cysts are mainly located in the lungs and liver (Buttenschoen and Buttenschoen 2003). The cyst is composed of a fluid-filled vesicle containing two layers: a germinal layer and an outer acellular laminated layer (LL). The LL of *E. granulosus* is composed primarily of highly glycosylated mucins and calcium deposits of myo-inositol hexakisphosphate (Casaravilla *et al.* 2006, 2010; Diaz *et al.* 2011, 2015; Irigoin et al. 2004). In addition to the structural components of LL, host proteins are adsorbed to the surface of LL during infection. These proteins could play an important role in immune evasion, potentially modulating the host's immune response and

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Corresponding author:
C. Touil-Boukoffa;
Email: touilboukoffa@yahoo.fr

thus promoting parasite survival. The LL fulfills two crucial functions: acting as both a mechanical and immunological barrier against the host's immune defenses (Diaz *et al.* 2011; Zeghir-Bouteldja and Touil-Boukoffa 2022). The survival and persistence of the parasite in the host involves evasion strategies, which may be mediated by the LL. In fact, numerous studies have demonstrated the protective and/or immuno-regulatory effect of LL during many pathologies such as echinococcosis, IBD, and allergies (Amri and Touil-Boukoffa 2015; Benazzouz *et al.* 2023; Soufli *et al.* 2015).

Rheumatoid arthritis (RA) is one of these inflammatory autoimmune diseases showing increasing incidence in recent years (Almutairi *et al.* 2021). In Algeria, the prevalence of RA is 0.15 % of the population (Slimani and Ladjouze-Rezig 2014). RA is characterized by a chronic synovitis, which leads to irreversible cartilage and bone erosion and disability. The exact cause of RA is unknown; however, genetic and environmental factors are highly involved. Moreover, the immune system is known to play key role in the pathophysiology of RA (Firestein and McInnes 2017). In fact, the development and persistence of fully established rheumatoid synovitis requires the collaboration of a variety of immune cells (such as synovial cells, macrophages, dendritic cells, T cells, B cells, natural killer cells, and mast cells) and inflammatory mediators (such as cytokines and chemokines, matrix metalloproteinases (MMPs), and nitric oxide (NO)) (Itoh 2017; Yap *et al.* 2018).

Cytokines play an important role in RA (Arroul-Lammali *et al.* 2017) and are responsible for systemic and local inflammation, synovitis, and articular joint destruction. The characteristic inflammation of RA is due to the predominance of pro-inflammatory cytokines over anti-inflammatory cytokines. These pro-inflammatory cytokines induce the production of NO and MMPs. In contrast, anti-inflammatory cytokines inhibit these inflammatory mediators and induce the expression of arginase and tissue inhibitor of MMP 1 (TIMP-1) (Alam *et al.* 2017; Itoh 2017).

In the last few decades, there has been a revolution in the treatment of chronic inflammatory rheumatic diseases with the development of biological therapies that can inhibit molecular targets directly involved in the pathogenesis of RA. Biologic drugs include Infliximab (TNF-α inhibitor), Abatacept (T cell activation inhibitor), Rituximab (B cell-depleting monoclonal anti-CD20 antibody), Anakinra (human IL-1 receptor antagonist), and Tocilizumab (IL-6 receptor antagonist) (Lin *et al.* 2021). However, despite the considerable efficacy of some of these biological agents (Kerschbaumer *et al.* 2023), the proportion of patients achieving disease remission still remains low. Moreover, they have many serious side effects such as the increased risk of infections caused mainly by the suppression of the immune response (Guo *et al.* 2018; Ruderman 2012). In this context, the search for new drugs is urgently required.

The aim of our study was to investigate the ex vivo immunomodulatory effect of *Echinococcus granulosus*' laminated layer extract on NO and cytokines production, MMPs activity, and iNOS/NF-κB expression in mononuclear cells from RA patients with active disease (ARA). Moreover, we evaluated the impact of LL on the anti-inflammatory activities of drugs commonly used for RA patients in Algeria. Therefore, we first assessed the inflammatory response (NO production, MMPs activities, and iNOS/NF-κB expression) in patients with active and inactive RA in comparison to healthy subjects. Second, we examined the optimum LL concentration for use.

Patients and methods

Laminated layer (LL) separation and preparation

Hydatid cysts from human lungs were collected from the surgical departments of Rouiba and Djillali Belkhenchir hospitals (Algiers, Algeria). The laminated layer (LL) was extracted as described by Steers *et al.* (2001). First, the hydatid fluid was aspirated aseptically, and the hydatid membranes were subsequently washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) containing 1% penicillin-streptomycin (Sigma-Aldrich). After freezing the cyst wall overnight, the LL was carefully separated from the germinal layer, washed several times with PBS, and re-suspended in PBS containing a cocktail of protease inhibitors (Invitrogen, Life Technologies, Carlsbad, CA, USA). The LL was then homogenized and sonicated on ice for 10 s per minute until a particulate solution was obtained. The suspension was allowed to sediment overnight, followed by centrifugation at 12,000 rpm and 4°C for 30 min. The supernatant containing the LL extract was filtered through a 0.22 µm filter and treated with endotoxin removal resin (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the protein concentration of the LL extract was measured using the Bradford method, and the LL was stored at −80°C until its use (Amri and Touil-Boukoffa 2015).

Patients and blood samples collection

Our study included sixty-eight patients (19 men/ 49 women, mean age 46.43±14.41 years, mean disease duration 12.48 years) with rheumatoid arthritis according to the 2010 American College of Rheumatology (ACR)/ European League against Rheumatism (EULAR) classification criteria. They were recruited from the Rheumatology and Orthopedic departments of three hospitals in Algiers-Algeria (Ben Aknoun EHS, Bab-El-Oued, and Beni Messous). The demographics and clinical findings of patients are presented in Table 1. Patients with RA were divided into two groups according to their DAS28-ESR (Disease Activity Score 28 using the Erythrocyte Sedimentation Rate). Thirty-eight patients were in active stage of the disease (ARA) and thirty patients in inactive stage (IRA). Healthy volunteers were included as controls in this study (HC; n= 26). They were free from any inflammatory diseases or any other rheumatologic conditions.

All participants gave written informed consent, and the study was reviewed and approved by the local ethics committee of national agency of research development in health (ATRSS).

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Blood samples were collected on EDTA tubes and were immediately used for PBMC (peripheral blood mononuclear cells) separation.

Peripheral blood mononuclear cells (PBMCs) separation and culture

PBMCs were isolated from whole blood by Ficoll-Paque density-gradient centrifugation (Sigma-Aldrich). Cells were washed twice with sterile PBS (pH 7.4) (3000 rpm, 5 min) and resuspended in RPMI-1640 (Sigma-Aldrich) supplemented with 5% heat-inactivated Fetal Calf Serum (FCS, Gibco), 1% penicillin–streptomycin mixture

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Table 1. Clinical and demographic characteristics of RA patients and healthy controls

Characteristics	Active RA(ARA) (n=38)	Inactive RA(IRA) (n=30)	Healthy controls(HC) (n=26)
Age. yrs	46.66±15.9	46.14±13.61	31.5±10.44
Female. n (%)	32 (69.52)	17 (56.66)	22 (63.12)
Disease duration. yrs	12.11	12.96	/
Disease activity status. n (%)			
Remission	/	30 (100)	
Low	9 (23.68)	/	/
Moderate	19 (50)	/	/
High	10 (26.31)	/	/
ESR. (mm/hr)	35.36±24.27	15.71±14.65	/
DAS28ESR	3.66±0.76	2.03±0.87	/
Treatments. n (%)			/
NSAID	1 (2.63)	1 (3.33)	/
Corticosteroids	10 (26.31)	3 (10)	/
csDMARDs	23 (60.52)	12 (40)	/
bDMARDs	17 (44.73)	14 (46.66)	/

ARA: Active rheumatoid arthritis; IRA: Inactive rheumatoid arthritis; HC: Healthy controls; ESR: erythrocyte sedimentation rate; DAS-28: disease activity score 28; NSAIDs: nonsteroidal anti-inflammatory drugs; csDMARDs: conventional disease-modifying antirheumatic drugs; bDMARDs: Biological disease-modifying antirheumatic drugs. N: number; yrs: years

(Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich). The cell viability was estimated to be superior then 96% using the Trypan blue exclusion test.

The cells were cultured in 96-well microplates at a concentration 10^6 cells/mL. Cells from ARA and IRA patients and healthy controls were cultured without any stimulus (Arroul-Lammali *et al.* 2017). Cells from ARA patients were then stimulated with different concentrations of LL (50, 100, or 150 µg/mL). Moreover, PBMCs from ARA patients were stimulated with methotrexate (MTX; 0.5 µg/mL), anti-TNFα (10 µg/mL), anti-IL6 (10 µg/mL), anti-CD20 (10 µg/mL), alone or in combination with LL (50 µg/mL).

After 20 h of incubation at 37°C in a humidified atmosphere with 5% CO2, culture supernatants were collected and stored at −20°C until nitric oxide and cytokines measurement and gelatin zymography. The collected cells were tested for viability using the Trypan blue exclusion test to assess the cytotoxic effects of LL treatment. Immunofluorescence staining was also performed to analyze NF-κB and iNOS expression.

Nitric Oxide (NO) measurement

Nitric oxide (NO) levels in the culture supernatants were measured using a modified Griess method, as described by Touil-Boukoffa et al. in 1998. Briefly, 50 µL of sample was incubated for 20 min at room temperature in darkness with 25 µL of Griess B reagent (0.5% N-1-naphthylethylene diamine, prepared in 20% HCl) and 25 µL of Griess A reagent (5% sulfanilamide, prepared in 20% HCl) with 400 µL of distilled water. The absorbance was measured by spectrophotometer at 543 nm. The nitrite concentration was calculated from a standard curve prepared with sodium nitrite (NaNO2).

Cytokines measurements

Levels of TNF-α, IL-17A, IL-10, and TGF-β1 in culture supernatants were measured using an enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (RayBioandInvitrogen). Absorbance was measured at 450 nm using an ELISA plate reader (LABSYSTEM®). Cytokine concentrations (pg/mL) were quantified based on the standard curves. The assay sensitivity was 30 pg/mL for TNF-α, 4 pg/mL for IL-17A, 0.05 pg/mL for IL-10, and 8.6 pg/mL for TGF-β1.

Gelatin zymography

The levels of metalloproteinase activity in the supernatant samples were assessed by gelatin-substrate gel electrophoresis. Briefly, total proteins were electrophoresed on 8% sodium dodecyl sulfate (SDS) sulfate-polyacrylamide gels containing 0.2% gelatin. After electrophoresis, the zymograms were washed twice with 2.5% Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.4), 5 mM CaCl2, and 20 mM NaCl buffer for 18 h at 37° C. The gels were stained with Coomassie blue (R250) and destained. MMP activity was identified as clear bands against a blue background. MMP-2 and MMP-9 molecular weight were determined by comparison with the standards MMP9 (92 kDa) and MMP2 (72 kDa). The gels were scanned and analyzed using the ImageJ software. The density of each band is reported as the mean of three different measurements of the same gel for each sample run in triplicate.

Immunofluorescence (IF) staining

PBMCs cultured with or without LL were washed with PBS and fixed on glass slides in 4% formaldehyde at room temperature for 30 min. After rinsing three times with PBS, the cells were permeabilized with Triton X-100 (0.1%) and blocked with skim milk (5%) for 2 h, followed by overnight incubation with rabbit anti-iNOS primary antibody (diluted 1:50) or mouse anti-NF-κB p50 primary antibody (1:50, NF-κB p50 Polyclonal antibody, Invitrogen 513500). After three washes, cells were labeled with secondary anti-rabbit /anti-mouse antibodies conjugated with fluorescein isothiocyanate (FITC) (diluted 1:500) in the dark for 1 h. After the final washing, the nucleus was counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich). The slides were then cover slipped using PBS-glycerol (1:9 v/v). Finally, images were captured using a fluorescence microscope (Zeiss Axioskop 2, Germany) with a digital camera unit (Canon Power shot A640, Japan).

Statistical analysis

Results are presented as mean±standard error of the mean (SEM). When normally distributed, statistical differences were assessed using the t-test. Mann–Whitney U test was used for abnormally distributed variables. ANOVA was used for comparisons between more than two groups. Statistical analyses were performed using the GraphPad Prism® software (Inc., La Jolla, CA, USA). Quantitative analysis of fluorescence was performed using ImageJ software

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(Bethesda, MD). Differences were considered to be statistically significant at p values <0.05.

Results

Elevated iNOS/NF-Kb expression and MMPs activities in rheumatoid arthritis is associated with disease activity

As previously reported (Arroul-Lammali *et al.* 2017), NO levels were higher in ARA than in IRA and HC. To confirm that ex vivo NO production during RA is linked to iNOS and NF-κB upregulation, an immunofluorescence assay was performed on PBMCs after 20 h of culture. A significant increase in fluorescence intensity and number of positive cells for iNOS and the P50 subunit of NF-κB were noted in PBMCs from patients with ARA compared with those from IRA patients and HC (Figure 1A) as indicated by the Corrected Total Fluorescent Cells (CTFC; p<0.0001) (Figure 1B). Our results suggest that iNOS and NF-κB are overexpressed in ARA patient cells, and no detectable or significant difference in the fluorescence intensity was observed when comparing PBMC from IRA and control subjects (p≥0.05).

Zymography analysis of MMP activity showed the same profile as that of NO production. Indeed, the zymogram profile showed that all MMPs activities were higher in ARA patients than in IRA patients or healthy controls (Figure 2A).

Interestingly, densitometric analysis using ImageJ revealed that the relative activities of pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 in PBMCs from ARA patients were significantly higher than those from IRA patients (p<0.05, p<0.05, p<0.05, and p<0.01, respectively) and healthy controls (p<0.01, p<0.05, p<0.05, and p<0.01, respectively). However, no difference was observed between IRA patients and the healthy control group for all MMPs activities (p≥0.05) (Figure 2B).

Given the fact that there is no significant difference in NO production, iNOS/NF-κB expression, and MMPs activities between IRA patients and healthy subjects, we chose to carry out the rest of the experiments on PBMC from patients during the active stage.

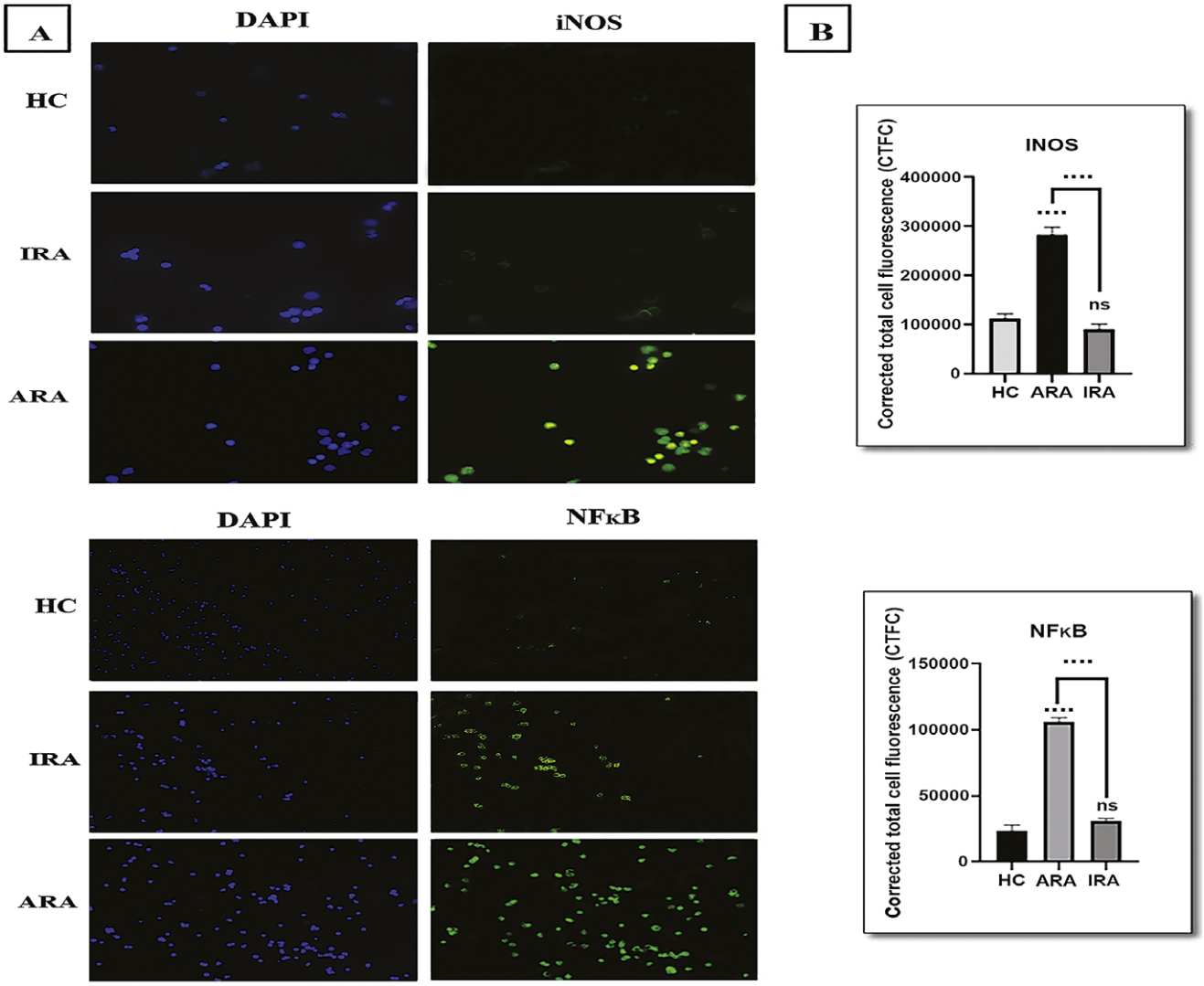

Figure 1. iNOS and NF-κB expression by PBMCs of rheumatoid arthritis patients (RA). ARA: RA patients with active disease. IRA: RA patients with inactive disease. HC: healthy controls. PBMC were cultured during 20 hours without stimulus as described in section 'Patients and methods'. DAPI: 4',6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate. **A)** Images represent arbitrarily selected areas (400. magnification) of the immunofluorescent staining analysis. **B)** Corrected Total Fluorescent Cells (CTFC) analysis of the represented groups expressed as mean ± SEM (ns: p≥0.05; ****: p<0.0001).

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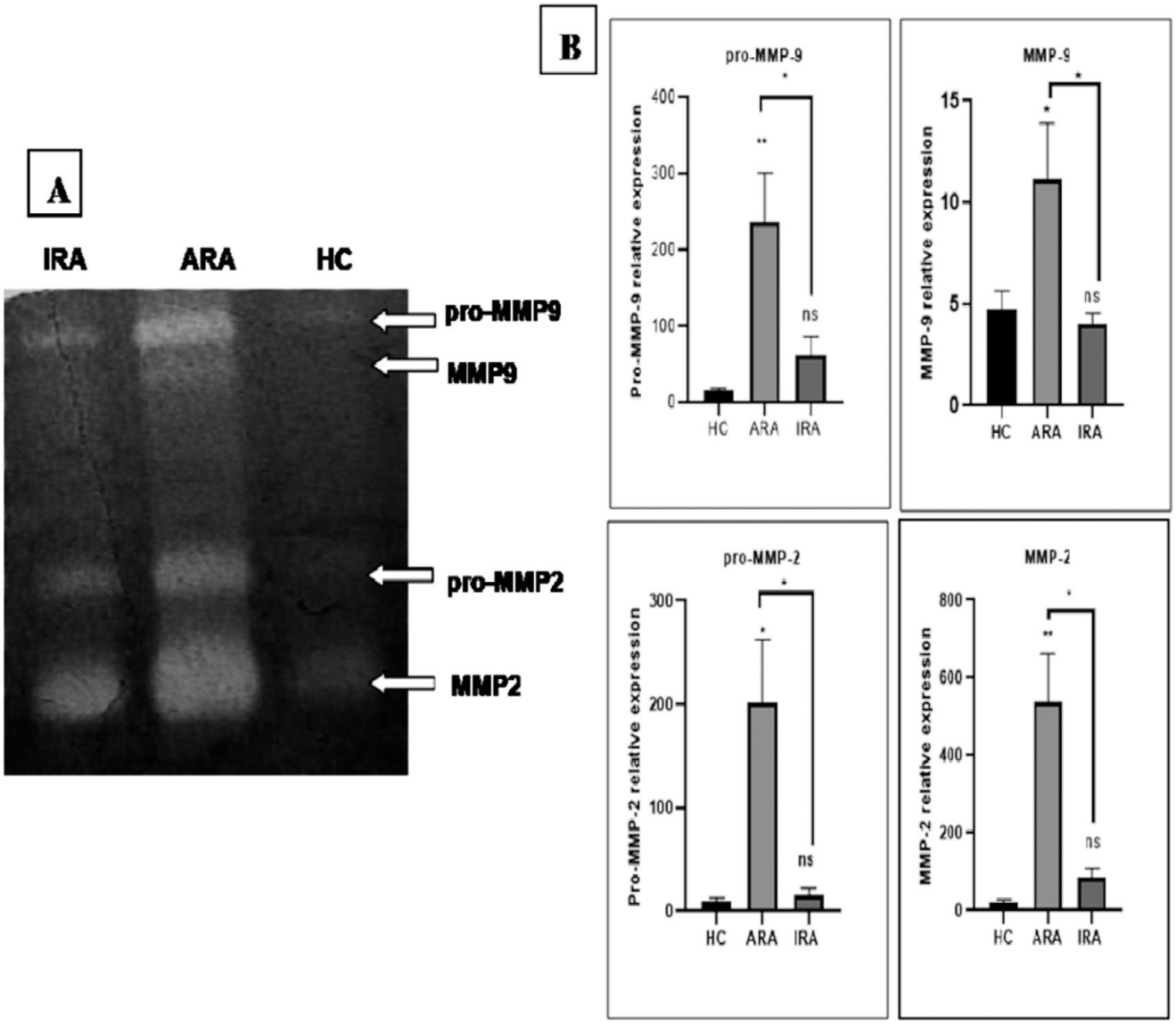

Figure 2. MMP-9 and MMP-2 activities in PBMCs of rheumatoid arthritis patients (RA). ARA: RA patients with active disease (n=38). IRA: RA patients with inactive disease (n=30). HC: healthy controls (n=26). PBMC were cultured during 20 hours without stimulus as described in section 'Patients and methods'. **A**) Zymogramme profile representative of MMP activities. **B**) Histogram presentation of MMPs expression levels after the densitometry analysis of Zymogramme with Image J software. All data are presented as the means ± SEM. (ns: p≥0.05; *: p<0.05; **: p<0.01).

Laminated layer reduces NO production by PBMC from active rheumatoid arthritis patients

To investigate the ex vivo immunomodulatory effect of LL on NO production, PBMCs from ARA patients were treated with serial concentrations of LL ranging between 50 and 150 µg/mL.

Our results revealed a significant decrease in NO production by PBMCs of ARA patients after treatment with LL. Therefore, NO levels decrease from 15.20±4.98 µM to 9.78±3.00 µM ($p<0.0001$), 11.13±4.04 µM ($p<0.01$), and 10.82±4.04 µM ($p<0.001$) in presence of 50, 100, and 150 µg/mL of LL, respectively (Figure 3).

To determine whether the concentrations of LL used are not cytotoxic, the viability of PBMCs was assessed after 20 h of culture with different concentrations of LL. Our results showed no significant difference in viability between untreated and treated cells, indicating that none of the doses of LL used (50, 100, 150 mg/mL) exerted any apparent cytotoxic effect on PBMCs.

We selected 50 µg/mL as the optimal concentration to reduce ex vivo NO production. It will be used to perform the rest of the culture stimulations.

Laminated layer reduces iNOS and NF-κB expression by PBMC from active rheumatoid arthritis patients

To determine whether LL modulates NO production via iNOS and NF-κB pathways, PBMCs from ARA patients were cultured with or without LL (50 µg/mL) for 20 h. The IF test results showed a significant decrease in the fluorescence intensity of iNOS and p50 subunit of NF-κB after treatment with LL. These observations suggest that LL modulates iNOS and NF-κB expression in ARA patients. Our results suggest that LL reduces ex vivo NO production during active RA by inhibiting the NF-κB signaling pathway (Figure 4).

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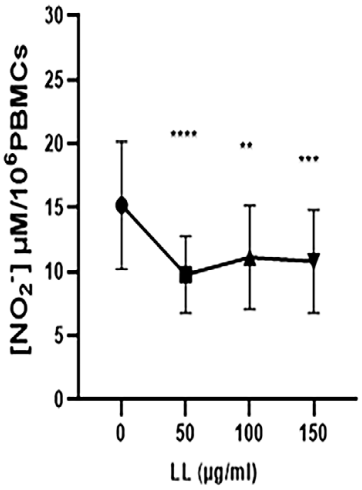

Figure 3. LL decreases NO production by PBMCs of RA patients with active disease (ARA). PBMCs were stimulated with laminated layer extract (LL) (50; 100 or 150 µg/mL) for 20 h as described in section 'Patients and methods'. All data are presented as the means ± SEM. (**:p<0.01; ***: p<0.001; ****: p<0.0001).

Laminated layer reduces MMPs activities by PBMC from active rheumatoid arthritis patients

The ex vivo effect of LL on MMPs activities in PBMC from ARA patients was investigated by gelatin zymography. The zymogram profile showed that anti-TNFα and LL reduced all MMPs activities (Figure 5A).

Interestingly, densitometric analysis by image J revealed that all MMPs activities were significantly downregulated after treatment of PBMCs with LL ($p<0.05$ for pro-MMP-9 and MMP9, and $p<0.01$ for pro-MMP2 and MMP2) (Figure 5B). Anti-TNF-α also decreased the expression of all MMPs ($p<0.05$).

Laminated layer reduces the production of pro-inflammatory cytokines and increases the production of regulatory cytokines by PBMC from active rheumatoid arthritis patients

To better understand the mechanisms by which LL exerts its inhibitory effects on NO and MMPs, we measured the levels of pro-inflammatory cytokines (TNF-α and IL-17A) and immune-regulatory cytokines (IL-10 and TGF-β1) in PBMCs' cultures treated with LL (50 µg/mL).

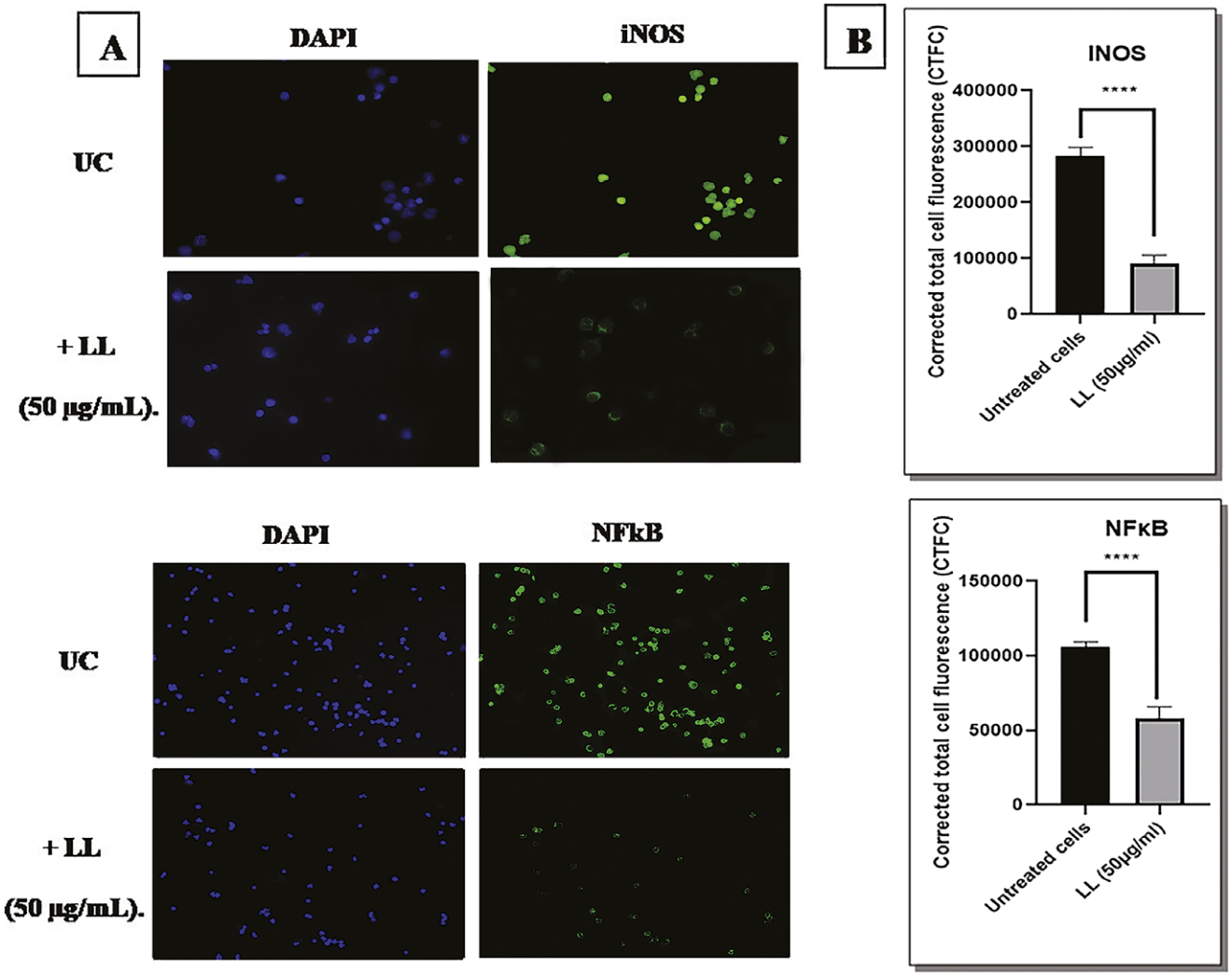

Figure 4. LL decreases iNOS and NF-κB expression by PBMCs of RA patients with active disease (ARA). UC: unstimulated cells. LL: laminated layer extract. PBMC were cultured during 20 hours without or with LL (50µg/mL) as described in section 'Patients and methods'. DAPI: 4',6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate. **A)** Images represent arbitrarily selected areas (400. magnification) of the immunofluorescent staining analysis. **B)** Corrected Total Fluorescent Cells (CTFC) analysis of the represented groups expressed as mean ± SEM (****: p<0.0001).

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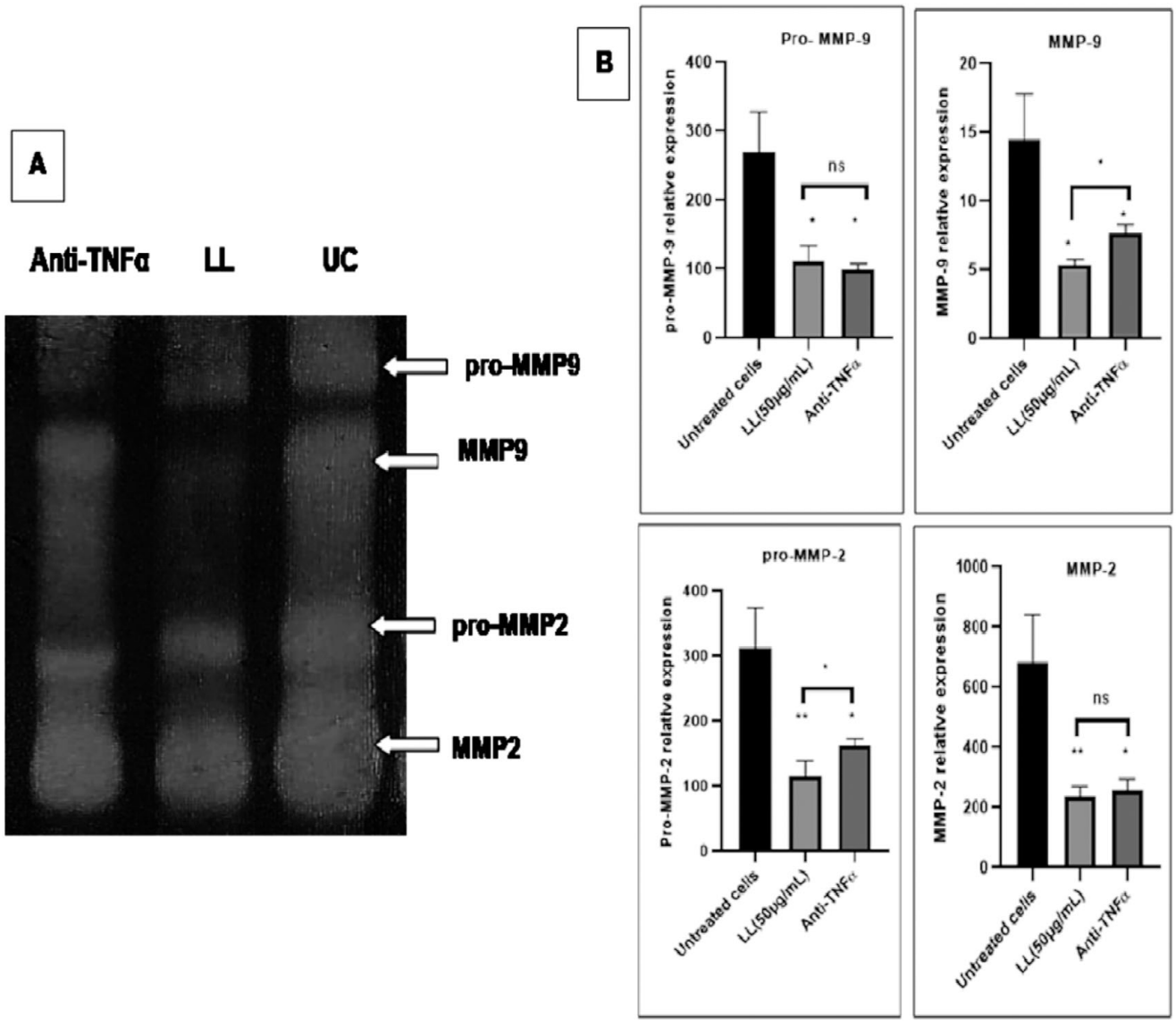

Figure 5. LL decreases MMPs activities by PBMCs from ARA patients. PBMCs were stimulated for 20h with LL (50 µg/mL) or Anti-TNFα (10 µg/mL) as described in section 'Patients and methods'. UC: unstimulated cells. LL: laminated layer extract. A) Zymogramme profile representative of MMP activities. B) Histogram presentation of MMPs expression levels after the densitometry analysis of Zymogramme with Image J software. All data are presented as the means ± SEM. (*:p<0.05; **:p<0.01).

Our results showed a significant decrease of TNF-α and IL-17A production by PBMCs after treatment with LL compared to the untreated cells (47.00±2.51 to 40.75±1.21 pg/mL; p<0.05, and 81.00 ±3.13 to 74.33±1.56 pg/mL; p<0.01, respectively). In parallel, LL treatment significantly increased IL-10 and TGF-β1 production by PBMCs of ARA patients compared to untreated cells (74.21±5.13 to 58.39±2.52 pg/mL; and 290.8±24.70 to 216.5±15.51 pg/mL, p<0.05). (Figure 6).

Laminated layer amplifies the inhibitory effect of usual drugs of NO production by PBMC from active rheumatoid arthritis patients

As rheumatoid arthritis is an inflammatory disease, biological drugs that suppress this inflammation are used. Synthetic (MTX) and biological (anti-TNFα, anti-IL-6, and anti-CD20) drugs are used in Algeria. Considering the significant inhibitory effect of LL on NO production, we investigated the effect of the combination of usual RA drugs and LL extract on NO production by PBMCs from ARA patients. Our results indicated that NO production was significantly reduced in the presence of anti-TNFα, anti-IL-6, anti-CD20 (p<0.0001), and MTX (p<0.05). Interestingly, this decrease was amplified when drugs (except for MTX) were used in combination with LL (50µg/mL) in comparison with unstimulated cells (p<0.0001 for anti-TNFα, anti-IL-6, and anti-CD20, and p<0.05 for MTX) or in cells stimulated with one drug (p<0.01 for anti-TNFα, anti-IL-6, and anti-CD20, and p≥0.05 for MTX) (Figure 7).

Discussion

In recent years, the role of the laminated layer (LL) of *Echinococcus granulosus* sensu stricto (Vuitton *et al.* 2020) as a physical barrier against immunological host responses has expanded to include key anti-inflammatory and immunomodulatory activities (Benazzouz *et al.* 2021, 2023; Diaz *et al.* 2011, 2023). Numerous studies have

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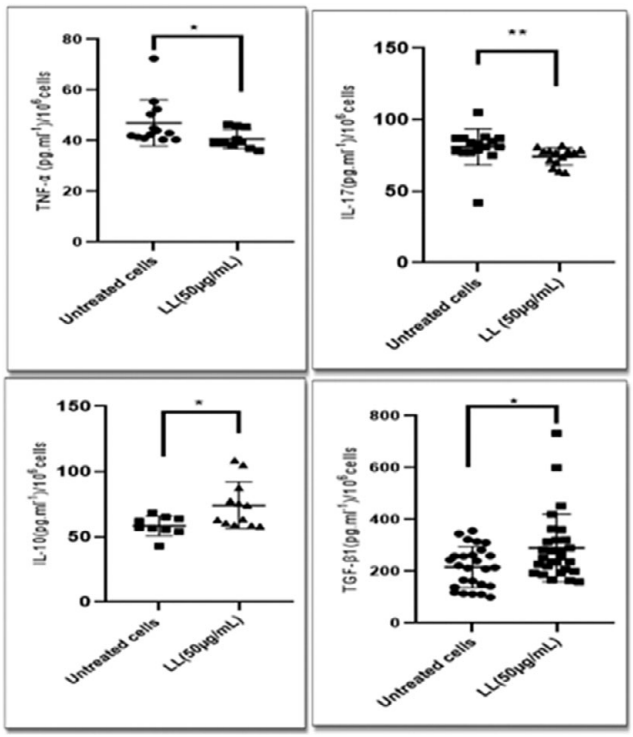

Figure 6. LL decrease TNF-α and IL-17A production and increase IL-10 and TGFβ1 production by PBMCs of RA patients with active disease (ARA). PBMCs were stimulated with laminated layer extract (LL) (50 µg/mL) for 20 h as described in section 'Patients and methods'. UC: unstimulated cells. LL: laminated layer extract. All data are represented as mean ± SEM with ANOVA-one way test. (*:p<0.05. **:p<0.01).

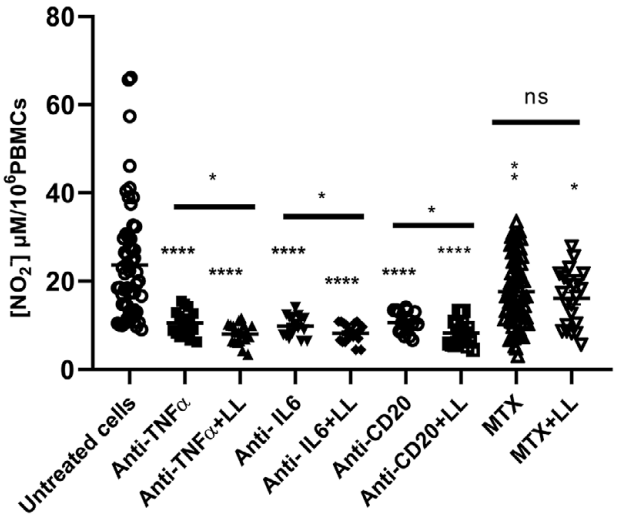

Figure 7. LL amplifies the effect of usual drugs used for RA on NO production by PBMCs from ARA patients. PBMCs were stimulated for 20h with Anti-TNFα (10 µg/mL). Anti-IL6 (10 µg/mL). Anti-CD20 (10 µg/mL). Methotrexate (MTX) (0.5 µg/mL) alone or with LL (50 µg/mL) as described in section 'Patients and methods'. UC: unstimulated cells. LL: laminated layer extract. All data are presented as the means ± SEM (ns:p≥0.05; *:p<0.05; ****: p<0.0001).

reported that LL is a potential candidate for regulating inflammation in many pathologies such as echinococcosis, IBD, and allergies (Amri and Touil-Boukoffa 2015; Benazzouz *et al.* 2023; Soufli *et al.* 2015). The results reported in the present study are in line with these data and suggest possible immunomodulatory and immunoregulatory effects of LL on immune cells from patients with RA in the active phase (ARA).

Rheumatoid arthritis is a chronic autoimmune disease characterized by the production of inflammatory cytokines and inflammatory mediators (such as NO and MMPs), which promote the development of synovitis and contribute to the worsening of patients' conditions, especially in the active phase of the disease (Itoh 2017; Nagy *et al.* 2010).

NO has been shown to be an inflammatory mediator of apoptosis in the rheumatoid joint (Van't Hof *et al.* 2000), suggesting that increased NO production plays an important role in the pathogenesis of RA. NO is produced by different cell types in inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils, and endothelial cells. Moreover, all cells express inducible nitric oxide synthase (iNOS) (Van't Hof and Ralston 2001). Increased NO production in RA patients could be related to increased NOS activity (Mäki-Petäjä *et al.* 2008). Interestingly, we showed that iNOS is more expressed in PBMCs from RA patients with active stage than those with inactive stage and healthy controls. Our results are supported by the study of St Clair *et al.* (1996), who demonstrated that iNOS protein is highly expressed in the blood mononuclear cells isolated from RA patients.

It is well known that iNOS expression is controlled by the nuclear factor-kappa B (NF-κB) signaling pathway in several diseases (Aktan 2004). In our study, PBMCs from IRA patients and healthy subjects expressed a lower fluorescence signal for the p50 subunit of NF-κB than in PBMCs from ARA patients. The importance of NF-κB in arthritis has been demonstrated in animal models where mice deficient in the p50 subunit are refractory to collagen-induced arthritis (Campbell *et al.* 2000). This increase in the DNA-binding activity of NF-κB has also been reported in both RA and murine collagen-induced arthritis (Han *et al.* 1998). These data indicate that NF-κB is one of the main inflammatory pathways involved in RA pathogenesis (Noort *et al.* 2015).

In addition to iNOS expression, NF-κB is a key regulator of matrix metalloproteinase (MMPs) gene expression in arthritis (Vincenti *et al.* 1998). Gelatinases MMP-2 and MMP-9 are key mediators of articular cartilage degradation. Indeed, these MMPs are abundant in the serum and synovial fluid of patients with RA (Tchetverikov *et al.* 2004). Interestingly, we noted high activities of pro-MMP9, MMP-9, pro-MMP2, and MMP-2 in PBMCs cultures during the active stage of the disease, suggesting a probable association between MMPs activity and clinical disease activity.

LL is a specialized extracellular matrix that has been extensively studied for its anti-inflammatory and immunomodulatory properties (Amri and Touil-Boukoffa 2015; Benazzouz *et al.* 2021, 2023; Diaz *et al.* 2011, 2022; Soufli *et al.* 2015). The biological activity of LL is attributed to its biochemical composition, which plays a role in parasite growth and protection from the host immunity. The LL structure is based on the fibrillar meshwork of mucins decorated with galactose-rich O-glycans. Moreover, LL includes calcium salt nanodeposits of myo-inositol hexakisphosphate (Insp6). Insp6 appears to be used by parasites to control complement-mediated inflammation (Diaz *et al.* 2022). Interestingly, studies have revealed the presence of host proteins within the LL, which persist even after multiple washing steps (Varela-Diaz and Colrorti 1973; Zeghir-Bouteldja and Touil-Boukoffa 2022). These tightly associated proteins, which are resistant to standard washing procedures, may play significant roles in parasite survival strategies and their interactions with the host immune system. Their persistence suggests that these proteins are implicated in immunomodulation, potentially contributing to the parasite's ability to evade or manipulate host immune

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responses (Díaz *et al.* 2022; Zeghir-Bouteldja and Touil-Boukoffa 2022).

Additionally, Andrade *et al.* (2004) reported that the *E. multilocularis* (*E.m.*) 14-3-3 protein present in LL appears to be one of the components responsible for the suppressive effect of this layer on the NO pathway. More studies are needed to evaluate the components of LL responsible for its effect.

In the present study, treatment of PBMCs cultures with LL showed very interesting and promising results. Indeed, NO levels decreased significantly after treatment with different LL concentrations, with the most pronounced effect observed at 50 µg/mL. Our data are consistent with the findings of numerous in vitro studies on mouse peritoneal macrophages (Amri and Touil-Boukoffa 2015; Steers *et al.* 2001), rat alveolar macrophages (Andrade *et al.* 2004), mouse splenocytes (Benazzouz *et al.* 2021), and PBMCs from asthmatic and allergic patients (Benazzouz *et al.* 2023). Similar results were reported in mouse models of DSS-induced colitis (Khelifi *et al.* 2017; Soufli *et al.* 2015).

Our results suggest that LL inhibits NO production in RA cells by regulating iNOS and NF-κB expression. The working concentration (50 µg/mL) of LL demonstrated a remarkable ability to downregulate iNOS and NF-κB expression. Similar results have been reported in intestinal tissues of mice with DSS-induced colitis (Soufli *et al.* 2015). Additionally, LL significantly reduced IL-17A and TNF-α production, as well as MMP activity, and enhanced IL-10 and TGF-β production in PBMCs from ARA patients. Ours results are consistent with those of Soufli *et al.* (2015) and Benazzouz *et al.* (2023).

According to our results, the inhibition of NF-κB p50 expression appears to be one of the molecular mechanisms by which LL suppresses these inflammatory mediators during active RA. In fact, it is well known that NF-κB signaling pathways are one of the pathways involved in the expression IL-17A and TNF-α (Akhter *et al.* 2023; Rex *et al.* 2023).

LL can inhibit NF-κB p50 expression by acting on the processing of the NF-kB precursor p105 to the p50 active subunit. This effect may occur through the action of LL particles, which suppress Akt phosphorylation in response to IL-4 (Seoane *et al.* 2016). Impaired Akt phosphorylation inhibits downstream signaling pathways, including the IKK/NF-κB pathway (Chen *et al.* 2002). It is well known that the processing of p105 to p50 involves the IκB kinase (IKK) complex, which is responsible for p105 phosphorylation and its subsequent ubiquitination to form p50 (Hinz and Scheidereit 2014; Salmeron *et al.* 2001).

Our findings demonstrate that LL has an anti-inflammatory effect during RA. In recent years, growing evidence has suggested that helminths and their derived molecules exert potent immunomodulatory and protective effects against autoimmune diseases, including RA, in animal models and in humans (Fleming 2013; Rostamirad *et al.* 2023; Varyani *et al.* 2017). Pearson and Taylor (1975) first reported that *Syphacia oblevata* infection reduced the incidence of adjuvant-induced arthritis in infected rats. Indeed, infection of mice with *Schistosoma japonicum*, *S. mansoni*, *Ascaris suum*, *and Hymenolepsisdiminuta* has demonstrated a protective effect against arthritis in various animal models (Bashi *et al.* 2016; Osada *et al.* 2009). These helminths act by modulating the balance of pro- and anti-inflammatory cytokines, leading to the reduced production of Th1 cytokines (Deepak and Goyal 2015) and elevated production of Treg cytokines (Grainger *et al.* 2010), as well as the activity of alternatively activated macrophages (Espinoza-Jimenez *et al.*2010). Moreover, *Trichinella spiralis* antigens have an anti-arthritic potential by reducing IL-17 level and increasing IL-10 production and Treg Foxp3+ cells number (Eissa *et al.* 2016). Additionally, ES-62, the major product secreted by the rodent filarial nematode *Acanthocheilonema viteae*, has been shown to protect mice against collagen-induced arthritis (CIA) and can suppress the pro-inflammatory responses of PBMCs and synovial cells of patients with RA (Al-Riyami *et al.* 2013; McInnes *et al.* 2003).

These data suggest that LL may have a potential therapeutic effect on RA. LL extract can be used as an adjuvant to MTX (methotrexate) or bDMARDs (biological disease-modifying anti-rheumatic drugs). Our results demonstrate that NO production by PBMCs of ARA patients was considerably reduced in the presence of anti-TNFα, anti-IL-6, anti-CD20, and MTX alone. Our results are in agreement with those of Gonzalez-Gay *et al.* (2009), who showed that anti-TNF-α treatment decreased NO production in patients with severe RA. Moreover, MTX inhibits NO production (Cronstein and Aune 2020). Despite many pharmacological advances, current therapies for arthritic diseases have adverse side effects and limited effectiveness. Additionally, the proportion of patients achieving disease remission remains low. Therefore, new alternative treatments are urgently required. Interestingly, our results show that the reduction in NO production by bDMARDs was greater in association with LL.

In light of our results, we showed that PBMCs from Algerian patients with RA expressed high levels of inflammatory markers, such as NO, iNOS, and NF-κB, and significant activities of MMP2/9. Interestingly, we demonstrated that LL of *E. granulosus* has an immunomodulatory effect through the upregulation of IL-10 and TGF-β. In parallel, LL decreased the levels of potent pro-inflammatory molecules, such as NO, iNOS, NF-κB, IL-17, TNF-α, and MMPs. Therefore, the development of drugs derived from the LL of *E. granulosus* could be a potential candidate for modulating inflammation during RA due to its efficacy and cost-effectiveness. Further investigations are required to fully explore the safety aspects and elucidate the exact mechanisms of action.

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Competing interest. The authors declare none.

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