VETERINARSKI ARHIV 93 (5), 559-568, 2023 DOI: 10.24099/vet.arhiv.2212

# *Salmonella* Typhimurium secondary infection induced macrophages apoptosis NO-dependent during *Mycobacterium neoaurum* infection

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**REN, J., D. YU, Y. SUN, H. SONG, X. JIANG, C. WANG: *Salmonella* Typhimurium secondary infection induced macrophages apoptosis NO-dependent during *Mycobacterium neoaurum* infection. Vet. arhiv 93, 559-568 2023.**

**ABSTRACT**

*Mycobacterium neoaurum* (*M. neoaurum*) and *Salmonella* Typhimurium (*S.* Typhimurium) are important zoonotic pathogens, and both are intracellular bacteria, which can induce cellular immunity. Coinfection is prevalent worldwide, even more prevalent than single infection. This study aimed to investigate the effect of *M. neoaurum*/*S.* Typhimurium coinfection on the percentage property of C57BL/6 mice regulatory T cells (Tregs) and the immune activity of RAW264.7 cells. The secretion of TNF-α, IFN-γ, IL-1β, IL-12 and iNOS in RAW264.7 cells was determined by ELISA. The expression of $CD40^+$, $CD80^+$ and $CD86^+$ costimulatory molecules on the surface of macrophages was analyzed by flow cytometry. A Nitric oxide (NO) assay was used to detect the production of NO in RAW264.7 cells. The apoptosis of RAW264.7 cells was detected by flow cytometry. The results showed that macrophage expressed a large number of cytokines and surface costimulatory molecules to enhance immune activity. *S.* Typhimurium secondary infection significantly increased the expression of iNOS and generation of NO, and *M. neoaurum*/*S.* Typhimurium-induced apoptosis was NO-dependent. Our data provide a theoretical basis for secondary infections by other pathogens after Nontuberculous Mycobacterium (NTM) infection, and lay a foundation for further research.

**Key words:** apoptosis; *Mycobacterium neoaurum*; nitric oxide; *Salmonella* Typhimurium.

## Introduction

*Mycobacterium neoaurum* (*M. neoaurum*) is a rapidly growing Nontuberculous Mycobacterium (NTM), which is widely distributed in the natural environment (JEON, 2018). The prevalence of NTM varies around the world, but overall, the prevalence and carrier rate of NTM are on the rise, with an estimated annual increase of 2.5%~8.0% (KOH, 2017). In general, NTM is in an asymptomatic colonization state in the body, so coinfection can easily occur (GARCIA et al., 2022).

*Salmonella* Typhimurium (*S.* Typhimurium) is one of the most common bacterial pathogens worldwide, with a broad host range (STANAWAY et al., 2016). It occupies the second position in Europe and third in the United States in human salmonellosis reports (FERRARI et al., 2019). *M. neoaurum* and *S.* Typhimurium are important zoonotic pathogens, as intracellular bacteria, which can induce cellular immunity. Immunocompromised groups are more susceptible to both (TADESSE, 2014).

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ISSN 0372-5480
Printed in Croatia

Coinfection refers to the simultaneous infection of the same host by more than one pathogen (ZHU et al., 2017). Coinfection is prevalent worldwide, even more prevalent than single pathogen infection (MICHAEL et al., 2004). After infection with NTM, except for a very small number of immunocompromised people who become ill immediately after infection, the vast majority of infected people will enter a long-term stable persistent state of infection (BOOM et al., 2021), so it is easy to induce coinfection with multiple pathogens. In tests, *S.* Typhimurium secondary infection significantly affected the process of *Schistosoma japonicum* (*S. japonicum*) infection, reduced the mortality of mice infected by *S. japonicum,* and alleviated the damage caused by *S. japonicum* to the host to a certain extent (ZHU et al., 2017). Coinfection with influenza A virus (IAV) after infection with *Mycobacterium tuberculosis* (*Mtb*), blocking IL-10 signaling, reduced the bacterial load in coinfected mice to a level comparable to *Mtb*-only-infected mice. The IL-10 signaling pathway is a major pathway for enhancing susceptibility to *Mtb* during IAV coinfection (RING et al., 2019).

At present, the research on pathogenic bacterial infectious diseases, including *S.* Typhimurium, has mainly focused on the bacterial infection process, the host immune responses, and the pathological changes in some organs (BAKALETZ, 2017). WANG et al. (2020) reported that the weight of mice decreased and mortality increased significantly after secondary infection with *S.* Typhimurium during *M. neoaurum* infection (WANG et al., 2020a). Therefore, on the basis of WANG et al. (2020a), we performed coinfection experiments on RAW264.7 cells. The aim of this study is to determine the effect of *M. neoaurum*/*S.* Typhimurium coinfection on the secretion levels of inflammatory factors, NO production and the apoptosis rate of macrophages, to provide a theoretical basis for analyzing the immune damage caused by coinfection with other pathogenic bacteria after infection with NTM.

## Materials and methods

*Bacterial strains. M. neoaurum* was isolated from submandibular lymph nodes from cattle, according to the method described in the previous study (WANG et al., 2020a), preserved in our laboratory and cultured in 7H9 liquid medium. *S.* Typhimurium ATCC 14028 was kindly provided by the Jilin Provincial Engineering Research Center of Animal Probiotics (Jilin Agricultural University) and was cultured in LB medium at 37°C.

*Cell culture.* Murine macrophage RAW264.7 cells line were provided by ATCC, Rockville, USA. RAW264.7 cells were cultured in RPMI-1640 medium (100μg/ml streptomycin, 100U/ml penicillin and 10% FBS) at a density of $5\times10^5$ cells/2ml/well in 6-well cluster plates (Corning, NY, USA). Adherent macrophages were inoculated with *M. neoaurum* or *S.* Typhimurium for 6h, or infected with *M. neoaurum* for 6h and secondarily infected with *S.* Typhimurium for 6h at MOI=10.

*ELISA*. After infection, the cultures were centrifuged at 335g/min for 10min at 4°C and the cell supernatant collected in sterile tubes. According to the manufacturer's instructions, the expression changes of TNF-α, IFN-γ, IL-1β, IL-12 and iNOS cytokines in RAW264.7 cells were detected. The ELISA kits were purchased from Shanghai Lengton Bioscience.

*Flow cytometry.* According to the method described in the previous study, single-cell suspensions of RAW264.7 macrophages were prepared (YANG et al., 2017). Cell suspensions were assigned equally to tubes with $1\times10^5$ cells per tube. RAW264.7 cells were stained respectively with monoclonal antibody FITC anti-CD40, FITC anti-CD80 and PE anti-CD86, according to the manufacturer's instructions. All antibodies were purchased from BD Pharmingen. Samples were acquired on an Accuri C6 flow cytometer (BD Biosciences, USA), and analyzed using FlowJo 7.6.0 software (Tree star, Ashland, OR).

*RAW264.7 apoptosis.* Cells were collected by centrifugation and washed in PBS twice. The cells were suspended with 200-300μL 1×Annexin V Binding Buffer and stained according to the manufacturer›s instructions (Annexin V/propidium iodide Apoptosis Detection Kit; BD Pharmingen). The cells were analyzed using an Accuri C6 flow cytometer (BD Biosciences, USA) and FlowJo 7.6.0 software (Tree star, Ashland, OR).

*Nitric oxide analysis.* The amount of nitrite in the supernatant of the cell cultures was measured using a nitrate/nitrite assay kit (Sigma Aldrich, code No. 23479). Briefly, first the nitrite, nitrate+nitrite calibration curve, and the sample solution were prepared, after which, 80μL of the sample solution and an additional 20μL of buffer solution. Then, 50μL of Griess Reagent A [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid] was added to each well, and the plate was incubated for 5min. Next, 50μL of Griess Reagent B was added, and the plate was incubated at room temperature for 10min. Finally, the absorbance of each well was measured at 540 nm with a microplate reader. [Nitrite]=[Nitrate+Nitrite]−[Nitrate] was the formula for calculation of the nitrite concentration in the sample solutions.

*Statistical methods.* All data were analyzed statistically by the one-way ANOVA test (Tukey's multiple comparison test), using Graphpad prism 6.0 software. The level of significance was considered at $P<0.05$.

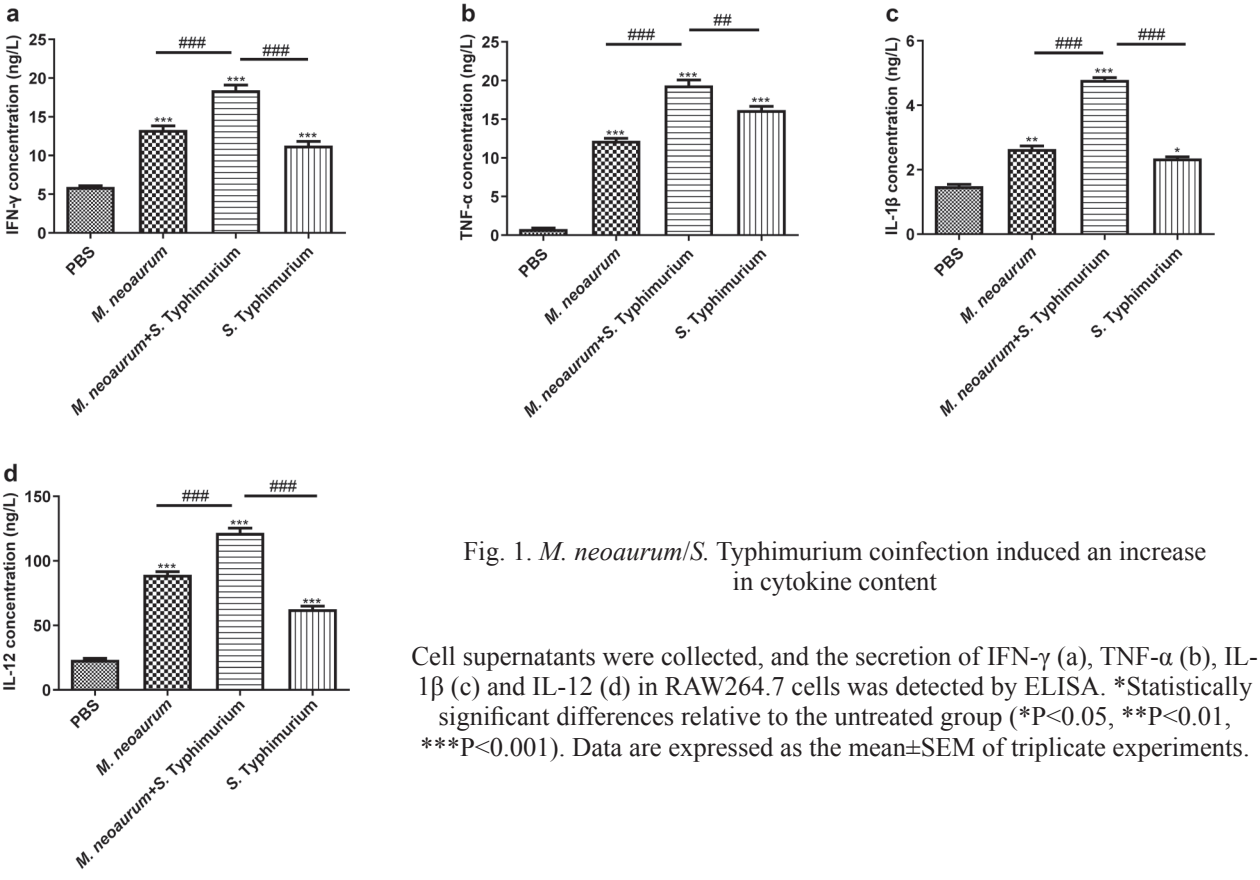


Fig. 1. *M. neoaurum*/*S.* Typhimurium coinfection induced an increase in cytokine content

Cell supernatants were collected, and the secretion of IFN-γ (a), TNF-α (b), IL-1β (c) and IL-12 (d) in RAW264.7 cells was detected by ELISA. *Statistically significant differences relative to the untreated group (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean±SEM of triplicate experiments.

## Results

*S. Typhimurium secondary infection induced increased cytokine secretion.* IFN-γ and TNF-α are critically involved in the control of NTM infection (YELLAYI et al., 2002). Therefore, we sought to determine the effect of *M. neoaurum/S.* Typhimurium coinfection on RAW264.7 cells' secretion of IFN-γ and TNF-α by ELISA. Fig. 1a-b show that the secretion of IFN-γ and TNF-α in the *M. neoaurum*+*S.* Typhimurium group were much higher than in the PBS group, the *M. neoaurum* group and the *S.* Typhimurium group

(P<0.001). Taken together, these data indicate that *S.* Typhimurium secondary infection could not impair NTM-specific macrophage responses and production of the proinflammatory cytokines IFN-γ and TNF-α, both of which are required for macrophage activation to control NTM replication. When the infection or inflammation is severe enough to affect an organ, macrophages first release TNF-α, IL-1β and IL-12 against the stimulus (POLL et al., 2021). Therefore, we detected the secretion of IL-1β and IL-12 by RAW264.7 cells by ELISA. Fig. 1c-d show that *S.* Typhimurium secondary infection caused macrophages to secrete IL-1β and IL-12 in large quantities, indicating that *S.* Typhimurium secondary infection caused a stronger immune response.

*Macrophage surface marker molecules were significantly expressed.* The co-stimulatory molecules CD40, CD80 and CD86 are all expressed on the surface of macrophages and are representative (FREEMAN et al., 2009). Therefore, it was necessary to detect their expression levels to study the effect of *S.* Typhimurium secondary infection on macrophages. We used flow cytometry to detect the expression of surface stimulation molecules in RAW264.7 cells. As shown in Fig. 2, the quantity in the *M. neoaurum*+*S.* Typhimurium group was significantly higher than in the *M. neoaurum* group and *S.* Typhimurium group, indicating that *S.* Typhimurium secondary infection could effectively stimulate macrophages to express CD40 (Fig. 2a), CD80 (Fig. 2b) and CD86 (Fig. 2c) molecules.

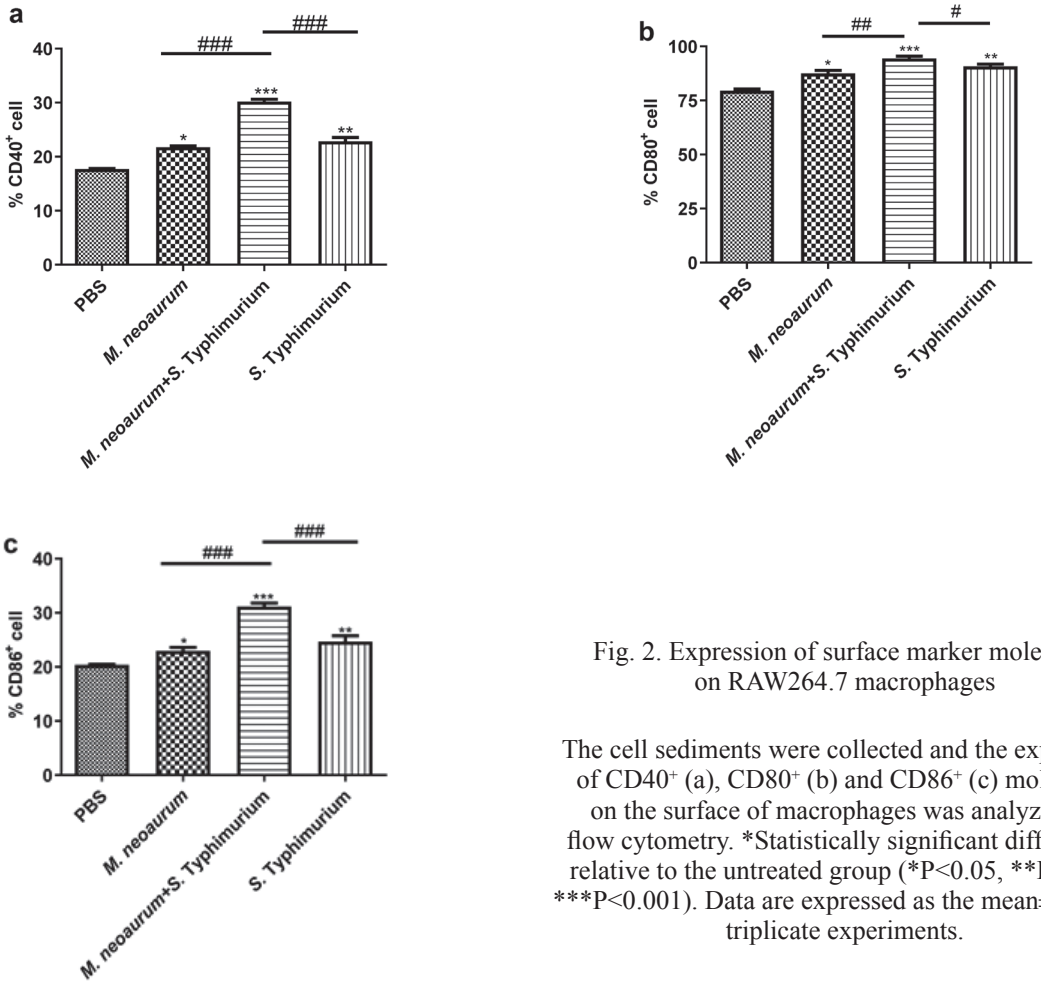


Fig. 2. Expression of surface marker molecules on RAW264.7 macrophages

The cell sediments were collected and the expression of $CD40^+$ (a), $CD80^+$ (b) and $CD86^+$ (c) molecules on the surface of macrophages was analyzed by flow cytometry. *Statistically significant differences relative to the untreated group (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean±SEM of triplicate experiments.

*S. Typhimurium secondary infection induced the expression of iNOS and production of NO in RAW264.7 cells.* IL-1β can activate iNOS to produce NO and ultimately induce apoptosis (STUART-SMITH, 1994). Therefore, we measured the production of iNOS, and the results showed that the production of iNOS increased in all infection groups, especially in the *M. neoaurum*+*S.* Typhimurium group (Fig. 3a). iNOS, once induced, could continue to synthesize a large amount of NO, which is involved in the elimination of bacteria, tumors, worms, fungi, viruses and the host's own toxicity by macrophages, especially to kill intracellular pathogens such as mycobacteria (DE GROOTE and FANG, 1995; NATHAN, 1995; XIE and NATHAN, 1994). Hence, the nitrate/nitrite colorimetric method was used to detect the variation trends of NO production after 6h treatment of RAW264.7 cells with *M. neoaurum* and *S.* Typhimurium. As shown in Fig. 3b, *S.* Typhimurium secondary infection induced a large amount of NO production.

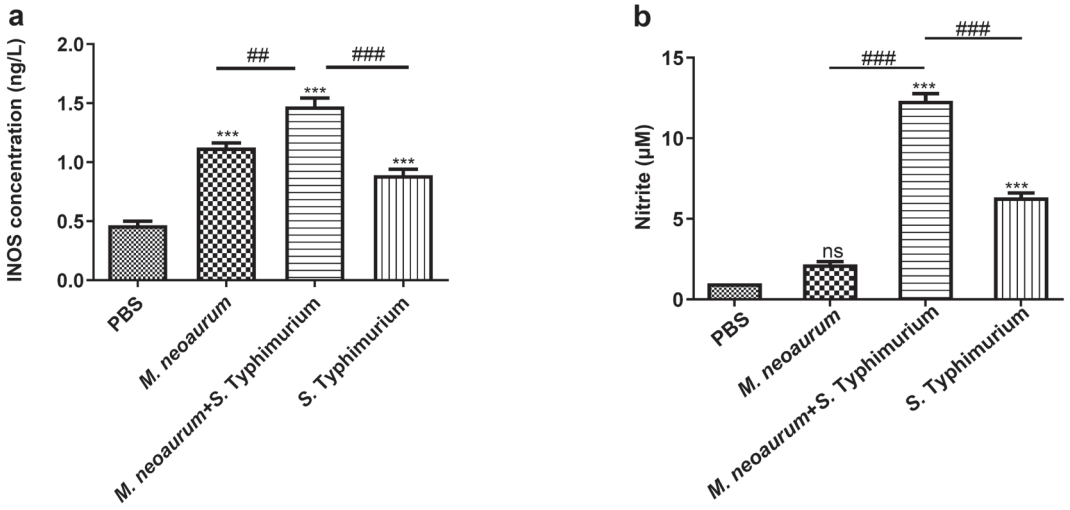


Fig. 3. *S.* Typhimurium secondary infection induced the expression of iNOS and production of NO

(a) The secretion of iNOS was detected by ELISA. (b) The production of NO was measured by nitric oxide assay. *Statistically significant differences relative to the untreated group (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean±SEM of triplicate experiments.

*S. Typhimurium secondary infection induced an increased apoptosis rate.* Apoptosis is particularly important for the body to inhibit or clear bacterial infection (PISTRITTO et al., 2016). To detect the apoptosis of macrophages after *M. neoaurum*/*S.* Typhimurium coinfection, we incubated RAW264.7 cells with *M. neoaurum* and *S.* Typhimurium, and detected apoptosis by flow cytometry. As shown in Fig. 4, the apoptosis rate reached nearly 30% after *M. neoaurum*/*S.* Typhimurium coinfection, which was about three times higher than the *M. neoaurum* group.

*Apoptosis induced by M. neoaurum and S. Typhimurium was NO-dependent.* These data demonstrated that *M. neoaurum*/*S.* Typhimurium coinfection promotes the generation of NO and increases the apoptosis ratio in RAW264.7 cells. NO is a bactericidal reactive-free radical, and has been reported to be involved in the regulation of cell death. To explore whether NO plays a role in the apoptosis of macrophages induced by *M. neoaurum* and *S.* Typhimurium, RAW264.7 cells were left untreated or treated with an NO scavenger (Carboxy-PTIO) at a concentration of 200μM, or

an NO donor (SNP) at a concentration of 200μM for 1h, followed by infection with *M. neoaurum* and *S.* Typhimurium for 6h, and apoptosis incidence was measured by flow cytometry. Our results showed that NO scavenger treatment led to a significant decrease in apoptosis, while the NO donor significantly increased the apoptosis (Fig. 5).

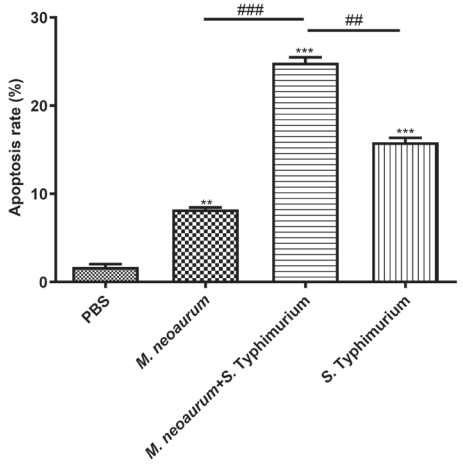


Fig. 4. *M. neoaurum*/*S.* Typhimurium coinfection promoted apoptosis in macrophages

The apoptosis rate was measured by flow cytometry. *Statistically significant differences relative to the untreated group (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean±SEM of triplicate experiments.

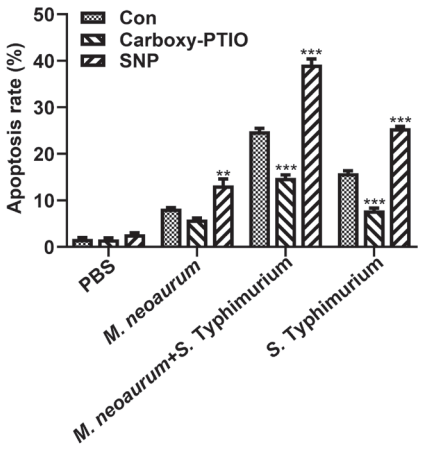


Fig. 5. Apoptosis induced by *M. neoaurum* and *S.* Typhimurium was NO dependent

RAW264.7 cells were left untreated (Con) or treated with an NO scavenger (Carboxy-PTIO) or an NO donor (SNP) for 1h, followed by infection with *M. neoaurum* and *S.* Typhimurium for 6h, then the apoptosis ratio was analyzed by flow cytometry. *Statistically significant differences relative to the untreated group (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean±SEM of triplicate experiments.

## Discussion

IFN-γ is a hallmark cytokine of type I helper T cells (Th1 cells), which has antiviral, immunomodulatory and antitumor properties (KAK et al., 2018). The role of IFN-γ is almost undoubted in mediating protection against tuberculosis (TB) (FLYNN et al., 1993). The hallmark of TB infection is the induction of cellular immunity and inflammation orchestrated by IFN-γ. TNF-α is mainly secreted by mononuclear macrophages (NI CHEALLAIGH et al., 2016). In addition to killing tumor cells, TNF-α is involved in immune regulation, fever, and inflammation (HORIUCHI et al., 2010). TNF-α involves the occurrence and development of many diseases, which can promote the production of various inflammatory factors by T cells, to promote the occurrence of inflammatory response (MEHTA et al., 2018). IL-1β is a potent pro-inflammatory cytokine that is crucial for host defense response to infection and injury (LOPEZ-CASTEJION and BROUGH, 2011). It is produced and secreted by a variety of cells and has been intensively studied within the cells of the innate immune system, such as monocytes and macrophages (BENT et al., 2018). IL-12 is mainly produced by B cells and macrophages, which could stimulate the proliferation of activated T cells and promote the differentiation of Th0 cells into Th1 cells (HILL and KOYAMA, 2020; LIBETTA et

al., 2007). Studies have reported that IL-12 could activate NK cells and T cells, and induce them to secrete a large amount of IFN-γ (ZHANG et al., 2020). The decrease in IL-12 levels weakens the effect on T cells and NK cells, and reduces the secretion of IFN-γ, which ultimately leads to further progression of the disease (WEHNER et al., 2011). GONDAIRA et al. (2015) found that *Mycoplasma bovis* (*M. bovis*) could induce the expression of important cytokines (TNF-α, IL-1β, IL-18, IL-2, IL-12, IFN-γ, and IL-6) in bovine peripheral blood mononuclear cells (PBMCs). Therefore, we examined the secretion of TNF-α, IFN-γ, IL-1β and IL-12 in macrophages after secondary infection with *S.* Typhimurium. The results showed that after secondary infection with *S.* Typhimurium, RAW 264.7 cells rapidly induced the massive secretion of TNF-α, IL-1β, IL-12 and IFN-γ cytokines. This is consistent with the strong induction of NO and IFN-γ secretion by co-infection of HIV-1 and *Mtb* studied in one previous piece of research (MUPFUMI et al., 2020).

Macrophages are important antigen presenting cells involved in regulating immune stimulation and tolerance, and they play an important role in the immune system (MUNTJEWERFF et al., 2020). The specific response of the body is that antigens are taken up by antigen-presenting cells, and processed intracellular and antigen presenting molecules MHC II and co-stimulatory molecules CD40, CD80 and CD86 are highly expressed (MBONGUE et al., 2017). Studies have shown that the expression level of CD80/86 could also change the movement ability of Treg cells, and thus regulate the cellular immune strength (GRACA et al., 2002; JONULEIT et al., 2001; ZHENG et al., 2004). It has been indicated that the upregulation of tumor-associated antigen expression with the benefit of CD40 activation enhances its immunogenicity, allowing immune cells to accumulate at tumor sites (LEE et al., 2013). We found that the expression of CD40, CD80 and CD86 costimulatory molecules on the RAW264.7 cell surface was higher after *S.* Typhimurium secondary infection, and all of them had statistical significance. The results indicated that the ability of macrophages to kill bacteria and cellular immune function were enhanced after *M. neoaurum*/*S.* Typhimurium coinfection.

Classically activated macrophages are characterized by a high ability to produce pro-inflammatory cytokines and NO by inducing the synthesis of iNOS (JUHAS et al., 2015). The basal activities of iNOS are generally very low, and in pathological conditions (inflammation, cancer, etc.) it will synthesize high concentrations of NO and inhibit cell growth or cell death by directly mediating monocyte macrophages to produce cytotoxic activity (WANG et al., 2020b). Studies have shown that NO is one of the key signals of tumor apoptosis (BONAVIDA and GARBAN, 2015). Previous studies have shown that the production of NO could lead to the oxidative stress injury of endothelial cells, induce their apoptosis, and directly damage the vascular endothelium, thus leading to the occurrence and development of Atherosclerosis (AS) (HUANG et al., 2017). In our study, 6 hours after secondary infection with *S.* Typhimurium, RAW264.7 cells secreted large amounts of iNOS, the production of NO increased dramatically, and the macrophage apoptosis rate was also significantly increased. We further explored the underlying molecular mechanisms of apoptosis induced by *M. neoaurum* and *S.* Typhimurium. The results indicated that NO scavengers could abrogate the apoptosis levels of macrophages induced by *M. neoaurum* and *S.* Typhimurium, while NO donors can promote apoptosis rate. This shows that apoptosis induced by *M. neoaurum* and *S.* Typhimurium depends on the production of NO. This is similar to the course of *Mtb* infection, overexpression of EBP50 (a scaffolding protein that controls the recruitment of iNOS in the vicinity of the phagosomes in macrophages) significantly increased the expression of iNOS and the production of NO, and the apoptosis induced by EBP50 depended on the production of NO (GUO et al., 2016).

Our study showed that *S.* Typhimurium secondary infection produced high concentrations of iNOS and NO in macrophages, effectively activating macrophage toxicity and improving the apoptosis rate of macrophages during *M. neoaurum* infection.

**Acknowledgements**

This study was supported by the national natural science foundation of China (31272566 and 31302124) and the science and technology research project of the education department of Jilin province (JJKH20200356KJ, JJKH20170315KJ).

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Received: 17 January 2023
Accepted: 22 may 2023

**REN, J., D. YU, Y. SUN, H. SONG, X. JIANG, C. WANG: Sekundarna infekcija bakterijom *Salmonella* Typhimurium tijekom infekcije bakterijom *Mycobacterium neoaurum* potiče apoptozu makrofaga koja ne ovisi o dušikovom oksidu. Vet. arhiv 93, 559-568 2023.**

**SAŽETAK**

*Mycobacterium neoaurum* (*M. neoaurum*) i *Salmonella* Typhimurium (*S.* Typhimurium) važni su zoonotski patogeni koji pripadaju unutarstaničnim bakterijama sa sposobnošću induciranja stanične imunosti. Koinfekcije navedenim bakterijama raširene su diljem svijeta, čak i u većoj mjeri od monoinfekcija. Cilj je ovoga rada bio istražiti učinak infekcije bakterijama *M. neoaurum* i *S.* Typhimurium na postotak regulacijskih mišjih C57BL/6 (Tregs) T-stanica te na imunosnu aktivnost stanica RAW264.7. Testom ELISA ustanovljeno je lučenje TNF-α, IFN-γ, IL-1β, IL-12 i iNOS u stanicama RAW264.7. Ekspresija kostimulacijskih molekula $CD40^+$, $CD80^+$ i $CD86^+$ na površini makrofaga analizirana je protočnom citometrijom. Za otkrivanje proizvodnje dušikova oksida (NO) u stanicama RAW264.7 primijenjen je test dušikova oksida. Apoptoza stanica RAW264.7 ustanovljena je protočnom citometrijom. Rezultati su pokazali ekspresiju velikog broja citokina i kostimulacijskih molekula na površini makrofaga kako bi se potaknula imunosna aktivnost. Sekundarna infekcija bakterijom *S.* Typhimurium znakovito je povećala ekspresiju iNOS-a i proizvodnju dušikova oksida, pri čemu apoptoza uzrokovana bakterijama *M. neoaurum* i *S.* Typhimurium nije ovisila o dušikovom oksidu. Naši podaci pružaju teorijsku osnovu za daljnja istraživanja i razumijevanje sekundarnih infekcija koju izazivaju drugi patogeni nakon infekcije netuberkuloznom bakterijom *Mycobacterium* (NTM).

**Ključne riječi:** apoptoza; *Mycobacterium neoaurum;* dušikov oksid; *Salmonella* Typhimurium