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IMMUNOSTIMULATORY ACTIVITY OF N.ACETYL D-GLUCOSAMINE VIA TLR$_2$ &TLR $_4$ MEDIATED MAPK,NF-κB SIGNALING PATHWAYS USING RAW264.7 MACROPHAGES

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Running Title: Immunostimulatory activity of NAG on RAW264.7 macrophages

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Abstract

Immunostimulatory agents are the one which stimulate the immune system and provides protection against various infectious diseases.Polysaccharides have proved to have beneficial role in the treatment of many pathological conditions.N-Acetyl D-glucosamine(NAG) is a monomer of polysaccharide,Chitin.The main aim of current work is to study immunostimultory effect of NAG on RAW264.7 macrophages,as the macrophages are the first cells which recognises the pathogen that cause infections.To this macrophages various test doses of NAG were administered during the study period and allowed to observe various parameters that support immunostimulatory effects.Results were obtained from triplicates were statistically analysed for significance.

Keywords: N-Acetyl D-glucosamine, immune system,immunostimulation,polysaccharides,

RAW264.7 macrophages

Introduction

Immunity is a speific host response against human pathogens to protect the human body from diseases caused by the entry of foreign pathogens.Immune response of the body is very important to combat various infectious diseases through the release of immunomodulators[1]. Among these immune cells, macrophages play a major role in the innate immune system. Innate immune system known to protect the human body from invading foreign pathogens by phagocytosis[2] . Moreover, macrophages activate the immune cells like T- lymphocytes and B –lymphocytes, which are responsible for the acquired immune response produced through the antigen –antobody reactions and the secretion of inflammatory mediators such as nitric oxide (NO), inducible Nitric oxide synthase (iNOS), interleukin-1β(IL1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα)[3]. Thus, macrophages play main role in both innate immunity response and acquired immunity.As the macrophages are involved all the types of immunities, activation of macrophages is an important step that can eventually block the development of infectious diseases[4]. Macrophages consist of a specific receptors known as Toll like receptors(TLR),binding of molecules to which results in activation of macrophages and release of inflamtory mediators through mitogen activated protein kinase(MAPK),nucler factor kappa B(NF- κB) and PI3K/AKT signaling pathways[5].

Polysaccharides are one of the crucial class of polymers with high nutritional value and industrial utility.They are not only the building blocks of living organisms but also responsible for several biological signals in cell-cell communication, immune recognition, and mitogenesis[6].In addition, unique physicochemical properties and biological functions of polysaccharides render them used as antioxidant, antitumor, wound healing, and immunostimulatory activities[7].According to the Food and Agriculture Organization (FAO),various indigestible plant polysaccharides such as cellulose, hemicelluloses, pectins, oligosaccharides, and gums are used as dietary fibers. Polysaccharides derived from plants and are non-toxic to humans when compared to the chemically synthesized polysaccharides[8]. Now a days,polysaccharides have been attracting the attention of human kind as immunoboosters.

Chitin is a polymer of N-acetylglucosamine, which is the most abundant polysaccharide in nature. The wide sources of Chitin are crustacean shells, insect cuticles, and fungal cell walls[9]. The low solubility of chitin limits its applications in various fields. Chitosan is the deacetylated product of Chitin which upon further hydrolysis produces chitosan oligosaccharides (COS) by chitosanases or non-specific glycosidases[10]. The COS are now commercialized based on their utility as cosmetics, animal husbandry, medicine, and food industries[11]. They have many therapeutical applictions as immunoregulatory[12], antioxidant [13], anti-tumor [14], anti-bacterial [15], antihypertensive [16], and anti-diabetes [17] activities, among others.The aim of present study is to investigate the immunostimulatory effect of NAG on RAW264.7 macrophages and its role in activation of various signaling pathways through TLR receptors.Since the macrophages play major role in immune response agianst to the entry of infectious agents,cell lines of macrophages were employed for the study.

Materials and Methods

Chemicals and reagents

N-acetyl D-glucosamine (NAG),Dimethyl sulfoxide (DMSO),),(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red , Griess reagent, TAK-242 (TLR4 inhibitor), C29 (TLR2 inhibitor), PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor) , The primary antibody such as p-p38, LC3, p62/SQSTM1, and β-actin and the secondary antibody such as anti-rabbit IgG, HRP-linked antibody were purchased from Sigma-Aldrich ,Banglore.

Cell culture

RAW264.7 cells (mouse macrophages) were purchased from American Type Culture Collection. They were cultured using Dulbecco's Modified Eagle medium (DMEM)containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at a temperature of 37 °C under a humidified atmosphere of 5% CO_2.

Estimation of cell viability and morphological analysis *of* RAW264.7 macrophages

The effect of NAG on the viability of RAW264.7 macrophages were assessed by the MTT colorimetric assay. This assay is used to detect the mitochondrial activity in living cells,in which tetrazolium penetrates into the living cells and forms formazan, purple in color crystals in the mitochondria. In this ,RAW264.7 macrophage cells were plated in 96-well culture plates at a concentration of 1×10^5 cells/ well and cultured for overnight. To this 100 μg/mL of NAG was added to the culture and they were incubated further for 24 h. The supernatants were discarded from the culture ,5 mg/mL of Tetrazolium salt solution was added to each of the well and incubated for 4 h .After incubation, the culture medium from all the wells were aspirated carefully . Then 100 μL of DMSO was added to each well to dissolve the formazan crystals. By using a microplate reader (Varioskan Lux, Thermo Scientific, Waltham, MA, USA)absorbance of each well was measured at 570 nm. To assess the effect of NAG on
morphology of mcrophages, the cells were photographed under an optical microscope,[18,19].

Measurement of NO production

Effet of NAG on NO production from RAW264.7 cells was determined by Griess assay. RAW 264.7 cells at a density of 1×10^5 cells/ml were incubated with various concentrations of NAG for 1 h and then exposed to LPS (1 μg/ml) for 24 h. After incubation, collected supernatants were reacted with Griess reagent (G4410, Sigma, St Louis, MO, USA)[20]. 100 μl of the supernatant and 100 μl of grease reagent were mixed and reacted at room temperature for 10 min, and absorbance was measured at 540 nm by using a microplate reader (Spectramax M2; Molecular Devices, CA, USA)[21].

Measurement of phagocytic activity

The effect of test compound on phagocytosis of RAW264.7 macrophages were determined using Neutral red uptake assay. RAW264.7 cells were plated in a 6-well plate for 24 h and they were simultaneously treated with treated with different concentrations of NAG at 37 °C under a humidified atmosphere of 5% CO_2 for 24 h. Then, the medium was removed from the wells and cells were stained with 0.01% of Neutral red solution for 2 h. After 2 h, the RAW264.7 cells were washed thrice with phosphate buffer solution .Then the absorbed neutral red from RAW264.7 cells was eluted by treating them with a cell lysis buffer (ethanol:acetic acid = 1:1). The absorbance of eluted Neutral red was measured using UV/Visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea) at 540 nm [22,23].

Measurement of prostaglandin E2 production in RAW 264.7 macrophages

To measure the production of PGE2 ,RAW 264.7 cells at a density of 1×10^5 cells/ml were incubated with various concentrations of NAG for 1 h and then exposed to LPS (1 μg/ml) for 24 h. After incubation, collected supernatants were used to determine the levels of PGE2 by selective ELISA kits according to the manufacturer's instructions[24,25].

Measurement of ROS Production

Nitro blue tetrazolium (NBT) reduction test was used to measure ROS production of RAW264.7 macrophages[26]. In this test, the RAW264.7 macrophages were plated on 96-well plates at a density 2 × 105 cells/mL and cultured for 24 h. Then the wells were incubated for 4 h with different cincentrations of NAG, or 1 μg/mL LPS to RAW264.7 cells,which were pretreated with 1 × PBS, or 100 μg/mL of NAG for 24 h. The cells were further incubated at 37 ◦C for 0.5 h with 200 μL of 1 × PBS, containing 0.1% of NBT (Sigma, USA) and 2 μg/mL phorbol 12-myristate 13-acetate (PMA). The reaction was terminated by adding 80 μL of 70% methanol. The cells were air-dried, and the blue formazan was dissolved in 120 μL of 2M KOH and 140 μL of DMSO. The absorbance was measured by spectrophotometer at 620 nm, using KOH/DMSO as the blank[27].

Reverse transcription polymerase chain reaction (RT-PCR)

The mRNA from RAW264.7 mcrophages was isolated using a RNeasy Mini Kit,as well as the synthesis of cDNA from the isolated mRNA was carried out by Verso cDNA Kit[28]. By using a PCR Master Mix Kit and the primers, amplification of the target gene was performed. The sequences of the primers used in this study were as follows: iNOS: forward 5′-ttgtgcatcgacctaggctggaa-3′and reverse 5′-gacctttcgcattagcatggaagc-3′, IL-1β: forward 5′-ggcaggcagtatcactcatt-3′ and reverse 5′-cccaaggccacaggtattt-3′, IL-6: forward 5′-gaggataccactcccaacagacc-3′ and reverse 5′-aagtgcatcatcgttgttcataca-3′, TNF-α: forward 5′-tggaactggcagaagaggca-3′ and reverse 5′-tgctcctccacttggtggtt-3′, GAPDH: forward 5′-ggactgtggtcatgagcccttcca-3′ and reverse 5′-actcacggcaaattcaacggcac-3′. The PCR results were obtained using agarose gel electrophoresis technique. The density of mRNA bands was calculated using the software UN-SCAN-IT gel version 5.1.[29]

Determination of Inflammatory Cytokine Production

RAW264.7 Macrophages were seeded in 96-well plates at a concentration of 5 × 105 cells/well. Then each well was treated with different concentration of NAG (0.1–25 μg/mL) for 24 h. The supernatant was measured using an ELISA kit(30) in accordance with the manufacturer's instructions . The level of each cytokine was determined using a standard curve obtained from the reaction of a standard substance[31].

Immunoblotting Assay

In this assay procedure,RAW 264.7 macrophages were cultured in 60-well cell culture plates at a density of 2×10^6 cells/well. Then they were treated with different concentrations of NAG. After treatment, the cultured cells were washed with phosphate buffer solution (PBS) and they suspended in a homogenizer containing lysis buffer. After centrifugation at 15,000 rpm and 4 ◦C for 15 min, the supernatant was collected. The concentration of protein was determined using DC protein assay kit . Proteins were separated by 6–15% polyacrylamide gel and transferred to the nitrocellulose (NC) membrane[32]. The protein-transferred membrane was blocked in 0.05%/Tris-buffered saline containing 5% skim milk powder for 1 h and then reacted with primary and secondary antibodies. The blots were developed using an enhanced chemiluminescence kit. The expression of each protein was quantified using image J software. After three repeated experiments, the average was taken and expressed[33].

Effect of NAG on the production of inflammatory molecules in RAW264.7 cells through TLR2 and TLR4

This experiment was performed to determine the role of toll like receptor 2 (TLR2) and toll like receptor 4 in the production of inflammatory molecules from NAG treated RAW264.7 macrophages.In this, RAW264.7 macrophages were pretreated with with C29 (TLR2 inhibitor) and TAK-242 (TLR4 inhibitor)and co-treated with NAG. Then the level of inflammatory molecules were measured[34].

Effect of NAG on Inhibition of NF-κB or MAPKs using specific inhibitors

The RAW264.7 cells were loaded into 96-well plates (1×10^4 cells/well) and incubated overnight. Then RAW264.7 cells were treated with PD98059, SP600125, SB203580, BAY 11-7082 for 2 h, respectively. Then cells with specific inhibitors were treated with NAG (100 μg/mL) for 8 h, the other two groups were treated with complete medium or NAG(100 μg/mL). the IL-1β, IL-6, TNF-α and NO content in the culture supernatant were determined according operation guide of ELISA kit[35,36].

Statistical Analysis

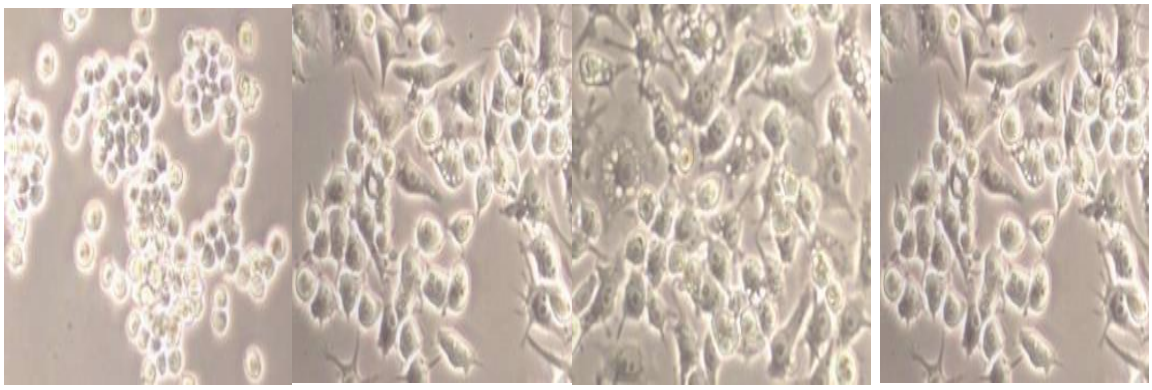
All data were expressed as the mean ± SD. All the experiments were conducted in triplicates and were representative of at least three separate experiments. Statistical analysis was made

by one-way AVOVA using the Student's t-test. p-values< 0.05 were considered to represent statistically significance.

Results

1. Estimation of effect of NAG on viability and morphological analysis of RAW 264.7cells

The effect of NAG on the viability of RAW 264.7 mcrophages was assessed by MTT assay. The 3-(4,5-dimethyltiazol-2 yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay showed that there was no obvious cytotoxic effects of NAG on RAW264.7 macrophages. Moreover, the administration of NAG at different doses(50,75,100µg/ml) has increased the size of RAW 264.7 cells and their surfaces became thick and rough. This indicated that NAG has activated RAW 264.7 cells (Fig. 1).



Control NAG(50µg/ml) NAG(75µg/ml) NAG(100 µg/ml)

Figure 1. Effect of different doses of NAG on morphology of RAW 264.7 macrophages

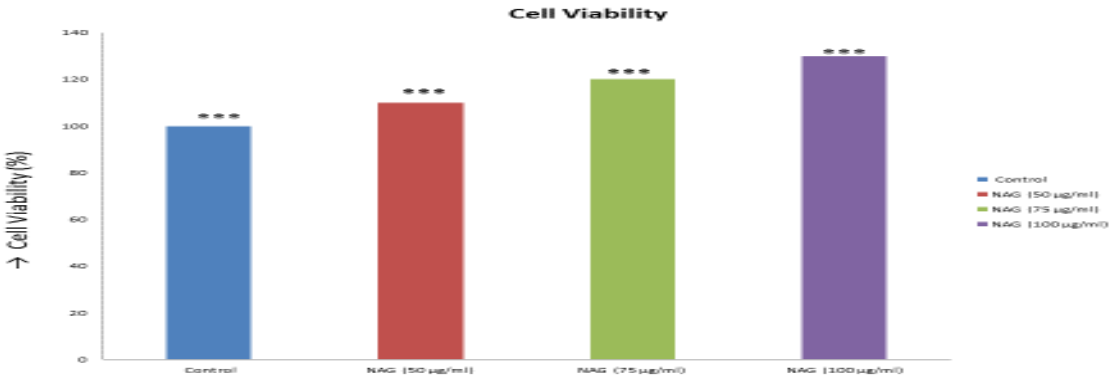


Figure 2. Effect of different doses of NAG on viability of RAW 264.7 macrophages

Effect of NAG on Production of NO from RAW 264.7 Macrophages

The effect of NAG on NO production from RAW 264.7 Macrophages was determined by Griess assay.NO is one of the inflammatory meditor produced from activated mcrophages.Administration of NAG has promoted production of NO from the activated RAW 264.7 Macrophages.It was also obsereved tht there ws a rise in expression of inflamatory molecules.

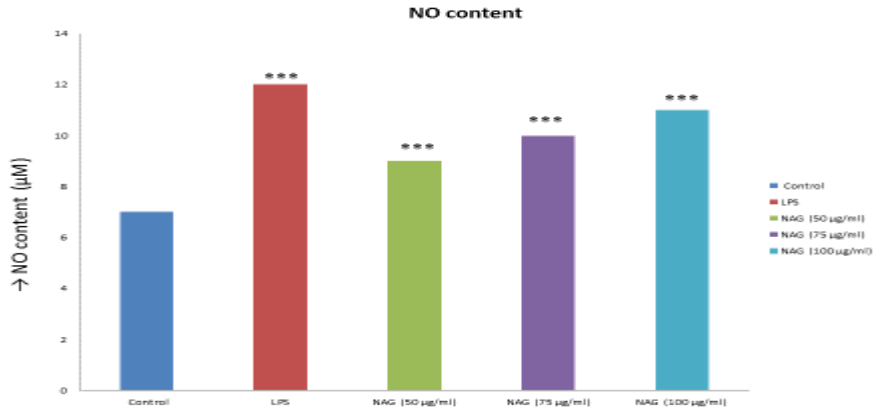


Figure 3. Effect of different doses of NAG on production of NO from RAW 264.7 macrophages

Effect of NAG on phagocytosis of RAW 264.7 Macrophages

Phagocytosis is the main function of macrophages in innate immune response. This is a crucial defense mechanism, which provides protection against invading pathogens an scavenging of necrotic cell debris in the immune system. Administrtion of NAG has improved the phgocytotic action of RAW 264.7 Macrophages**.**

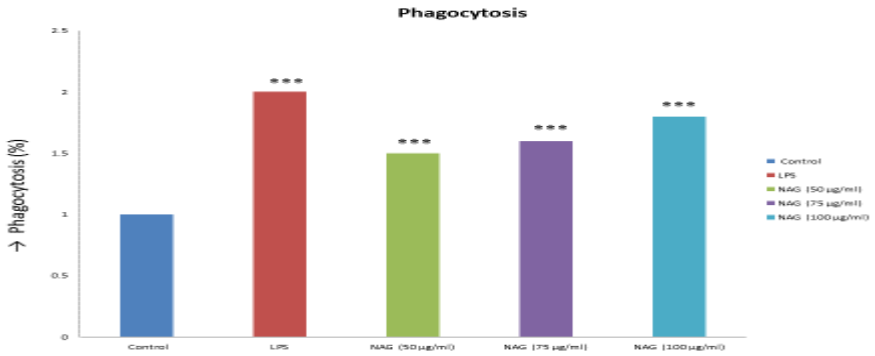


Figure 4. Effect of NAG on phagocytotic activity in RAW 264.7 macrophages considering 100%phgocytosis in control

Effect of NAG on the production of prostaglandin E2 in RAW 264.7 macrophages

In order to assess the effect of NAG on the release of PGE2, RAW 264.7 cells were treated with different concentrations of NAG for 24 hours. Following stimulation with NAG, the levels of PGE2 in the culture supernatants were determined by ELISA. It was observed that treatment of RAW 264.7 macrophages withdifferent concentrations of NAG has significantly increased PGE2 production compared to the control cells(unstimulted). LPS, which is a positive control, also induced the secretion of PGE2 from RAW 264.7 macrophages.

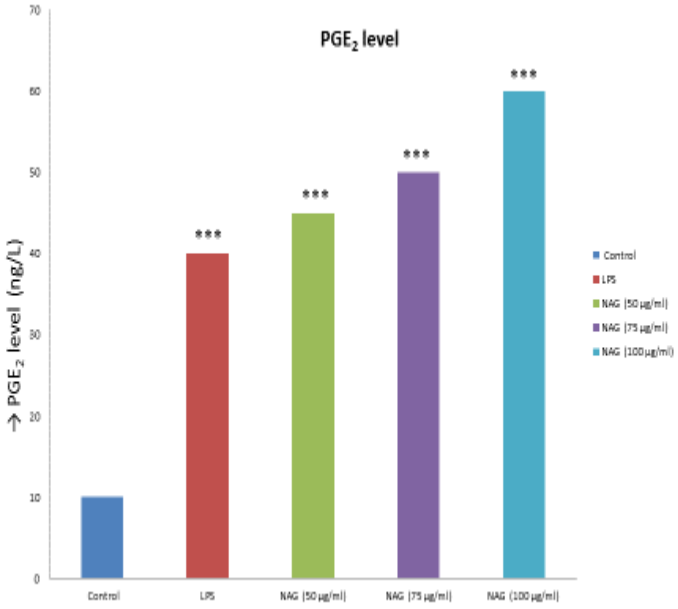
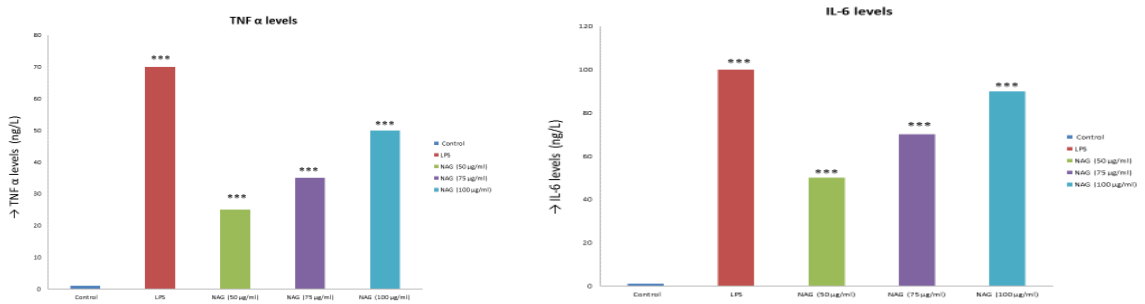


Figure 5. Effect of NAG on Induction of prostaglandin E2 production in RAW 264.7 macrophages.

Effect of NAG on Inflammatory Cytokine Production in RAW 264.7 macrophages

Activation of macrophages by different doses of NAG and LPS treatment has resulted in dose dependent increase in the levels of inflamatory cytokines TNF-α and ILs (IL-1β, IL-6, and IL-10) were significantly increased.



(a) TNF-α (b)) IL-6

Figure 6. Effect of NAG on production of inflammatory cytokines in RAW 264.7 macrophages such as (a)) TNF- α (b)) IL-6

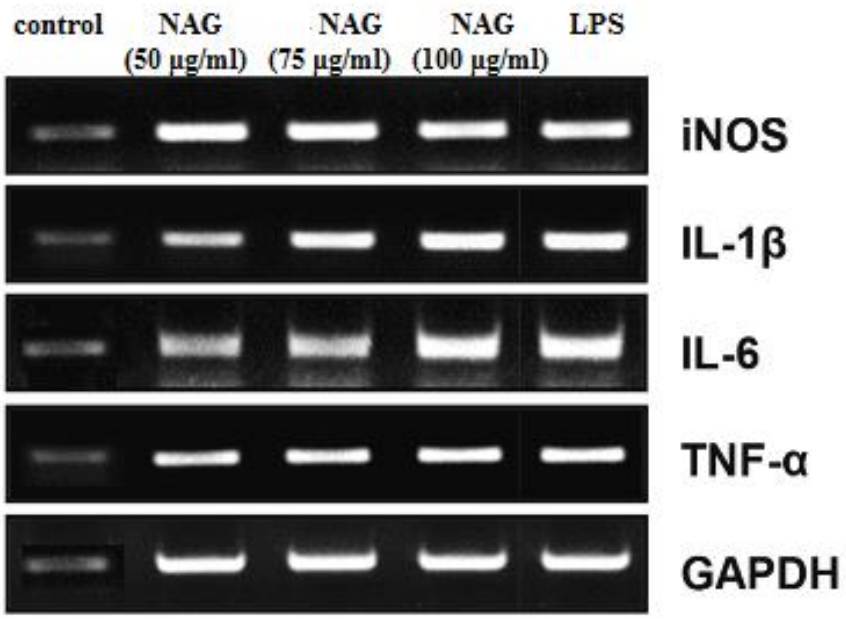


Figure 7. Effect of NAG on production of inflammatory cytokines in RAW 264.7 macrophages

Effect of NAG on the production of inflammatory molecules in RAW264.7 cells through TLR2 and TLR4

It has shown that production of NO was suppressed remarkably in TAK-242 inhibitor and NAG appliedmacrophages.But C29 treatment had no effect on HML-mediated NO

production. In addition, TAK-242 treatment inhibited the expression of various inflamatorymediaters such as iNOS, IL-1β, IL-6 and TNF-α in RAW264.7 cells treated with NAG. In addition, treatment of macrohages with C29 did not show any effect on NO production induced by NAG, it was shown that the expression of IL-1β, IL-6 and TNF-α were inhibited.

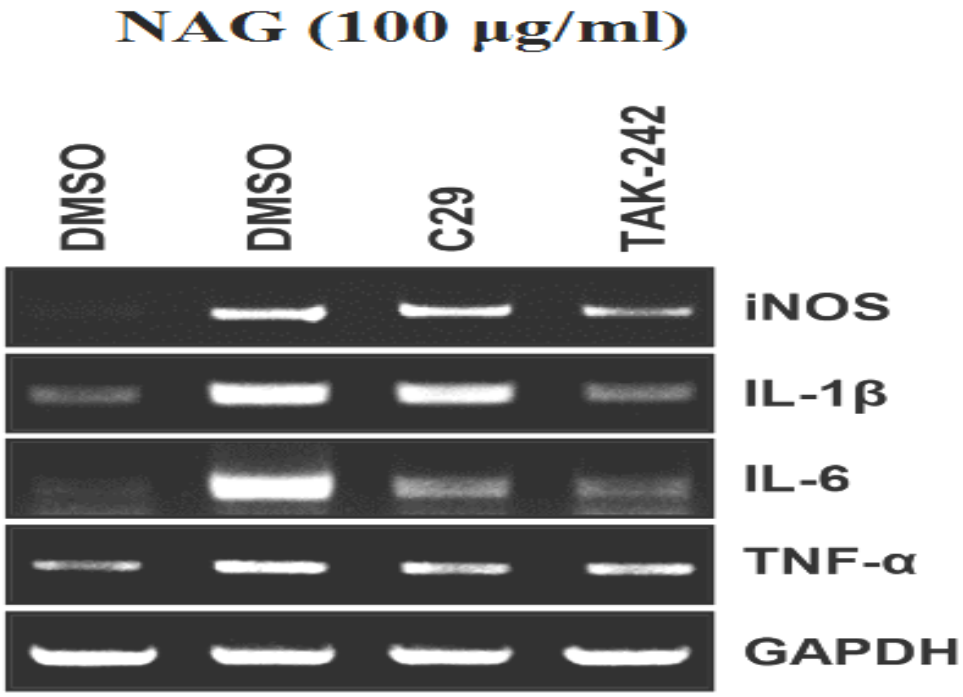


Figure 8:Effect of NAG on the production of inflammatory molecules in RAW264.7 cells through TLR2 and TLR4

Effect of MAPK signaling pathway on the production of inflammatory molecules in NAG-treated RAW264.7 cells

To know the effect of NAG on the production of inflammatory mediators in RAW264.7 cells through mitogen-activated protein kinase (MAPK) signaling pathway,macrophages were treated with MAPK signaling inhibitors and NAG.After treatment,the levels of inflammatory molecules produced by macrophages were measured.It was observed that ,first there was inhibition of NO production in presence of p38 inhibitor (SB203580) and JNK inhibitor(SP600125), but no effect on ERK1/2 inhibitor (PD98059).

It was also observed that, there was attenuation of iNOS expression by NAG in presence of SB230580 and SP600125 and IL-1β expression by was blocked by NAG with the treatment of 203580. Moreover IL-6 expression by NAG was inhibited in presence of SB203580 and TNF-α expression was slightly inhibited by NAG in presence of PD98059, SB203580 and SP600125 .

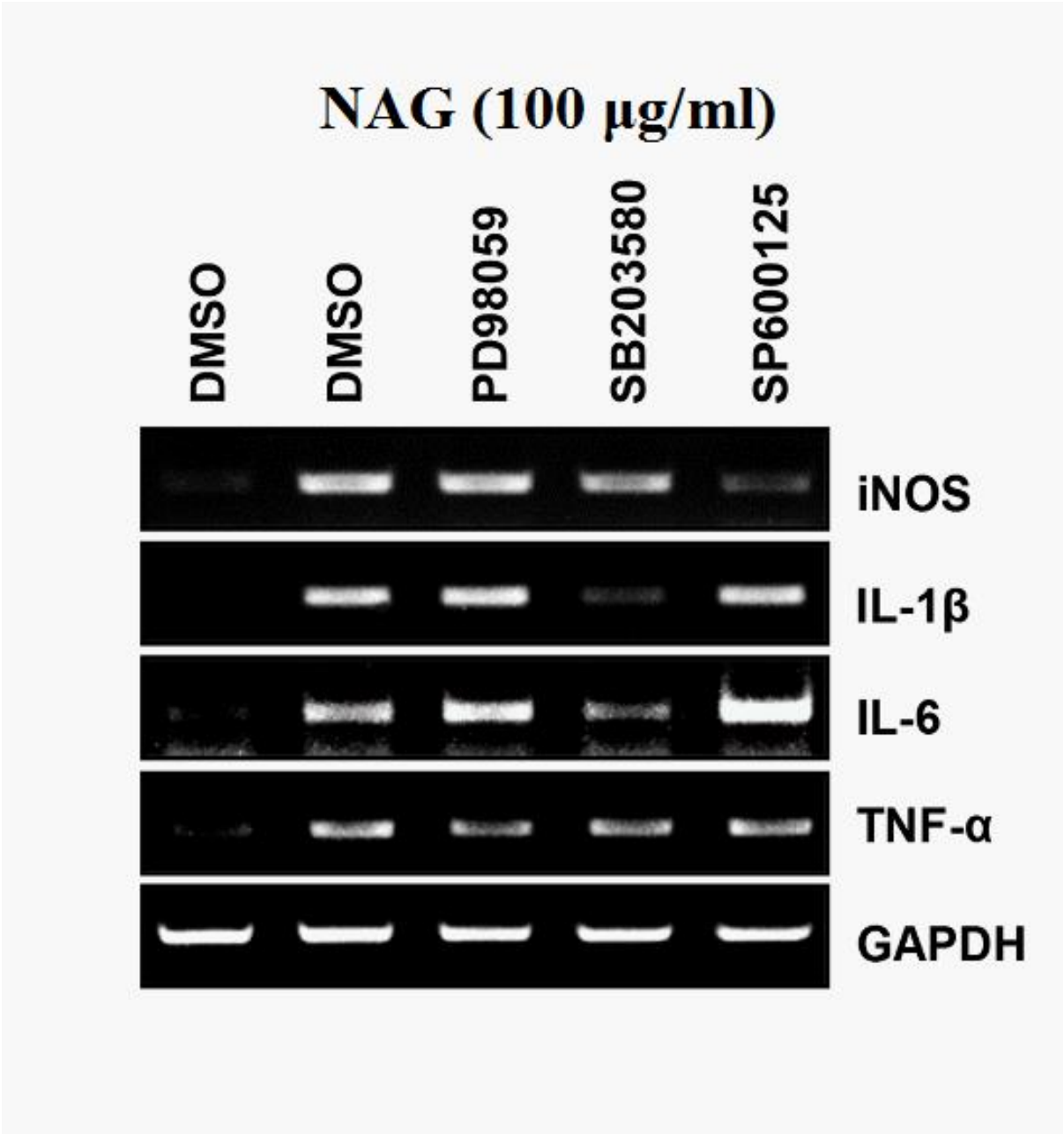


Figure 9: Effect of NAG on the production of inflammatory molecules through MAPK signaling pathway

Discussion

The prevalence of COVID-19 pandemic has rised the researcher's interest towards the immunostimulatory agents,which protect the body from various infections through the activation of immune system.In this present study we evaluated immunostimulatory activity of NAG on RAW264.7 macrophages.Treatment of NAG has increased the levels of various inflammatory cytokines such as TNF-α, IL-1β, NO and phgocytosis by interacting with the TOLL like receptors. Thus treatment of NAG proved immunostimulatory ctivity by activating macrophges.

Conclusions

In summary, the collective results of present study demonstrated that N-Acetyl D-glucosamine has shown immunostimulatory activity in RAW264.7 through activation of various signling pathways like MAPK and NF-κB.

Conflict of interest

There was no conflict of interest found by the authors.

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References

1. David D. Chaplin, Overview of the Immune Response. Journal of Allergy and Clinical Immunology. 2017 Dec (29): S3–23.

2. Daisuke Hirayama, Tomoya Iida, Hiroshi Nakase. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. International Journal of Molecular Science. 2018 Jan; 19(1): 92.

3. Guillermo Arango, Duqueand Albert Descoteaux· Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. Front Immunology. 2014(5):491.

4. Maurizio de Martino, , Luisa Galli and .Translational Insights into Pediatric Immune-Related Diseases. Pediatric Immunology 2019 August (27):12-23

5. Styliani Goulopoulou, Cameron G. McCarthy R. Clinton Webb, Stephanie W. Watts. Toll-like Receptors in the Vascular System: Sensing the Dangers Within. Pharmacology Reviews. 2016 Jan 68(1): 142–167.

6. Lovegrove A, Edwards C. H, De Noni I, Patel H, El S. N, Grassby T, Zielke C, Ulmius, L. Nilsson M , Butterworth P. J, Ellis P. R, Shewry P. R. Role of polysaccharides in food, digestion, and health :Critical Review. Food Science and Nutrition. 2017 Jan 22; 57(2): 237–253.

7. Salima T. Minzanova , Vladimir F. Mironov , Daria M. Arkhipova, Anna V. Khabibullina,

Lubov G. Mironova, Yulia M. Zakirova,Vasili A. Milyukov. Biological Activity and Pharmacological Application of Pectic Polysaccharides: A Review. Journal of Food Science Technology. 2012 Jun; 49(3): 255–266

8. Adrian T. Ting;Kenneth B. Marcu; James B. Bliska. Yue Zhang;Inhibition of MAPK and NF-κB Pathways Is Necessary for Rapid Apoptosis in Macrophages Infected with Yersinia ,J Immunol (2005) 174 (12): 7939–7949.

9. Daniel Elieh-Ali-Komi , Michael R Hamblin. Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. Int J Adv Res (Indore). 2016 Mar; 4(3): 411–427.

10. Michal Benedykt Kaczmarek,[1,2] Katarzyna Struszczyk-Swita,[1] Xingkang Li,[2] Miroslawa Szczęsna-Antczak,[1] and Maurycy Daroch[2.] Enzymatic Modifications of Chitin, Chitosan, and Chitooligosaccharides. Front Bio Engineering Biotechnology. 2019; 7: 243.

11. K. Jayathilakan, Khudsia Sultana, K. Radhakrishna, and A. S. Bawa. Utilization of by products and waste materials from meat, poultry and fish processing industries: a review. Journal of Food Science Technology2012 Jun; 49(3): 278–293.

12. Ani Grigorian , Lindsey Araujo, Nandita N Naidu. N-acetylglucosamine inhibits T-helper (Th1)/T-helper 17 (Th17) cell responses and treats experimental autoimmune encephalomyelitis. Journal of Biological Chemistry. 2011 Nov 18; 286(46):40133-41.

13. Tahia H. Saleem, Nagwa Abo El-Maali, Mohammed H. Hassan, Nahed A. Mohamed, Nashwa A. M. Mostafa, Emaad Abdel-Kahaar and Azza S. Tammam ;Comparative Protective Effects of N-Acetylcysteine, N-Acetyl Methionine,N-Acetyl Glucosamine against Paracetamol and Phenacetin Therapeutic Doses–Induced Hepatotoxicity in Rats

14. K. Hulíková, V. Grobárová, R. Křivohlavá ,A. Fišerová .Antitumor activity of *N*-acetyl-D-glucosamine-substituted glycoconjugates and combined therapy with keyhole limpet hemocyanin in B16F10 mouse melanoma model.Folia Microbiologica volume 55, pages528–532 (2010)

15. V.Blagodatskikh[a]Sergey N.Kulikov[b]Oxana V.Vyshivannaya[a]Evgeniya A.Bezrodnykh[a]Igor A.Yamskov[a]Vladimir E.Tikhonov[a] .Influence of glucosamine on oligochitosan solubility and antibacterial activity. Carbohydrate -research Volume 381, 15 November 2013, 28-32

16. Norbert Fülöp, Richard B. Marchase, John C. Chatham. Role of protein *O*-linked N-acetyl-glucosamine in mediating cell function and survival in the cardiovascular system. Cardiovascular Research. 2007 Jan 15; 73(2): 288–297.

17. Thi Ngoc Tran, Chien Thang Doan, Van Bon Nguyen, Anh Dzung Nguyen,San-Lang Wang. Anti-Oxidant and Anti-Diabetes Potential of Water-Soluble Chitosan–Glucose Derivatives Produced by Maillard Reaction. Polymers 2019, *11*(10), 1714.

18. ei Li, Qiufeng Zhang, Lihong Chen, Lihong Chen.Anti-inflammatory activities of Guang-Pheretima extract in lipopolysaccharide-stimulated RAW 264.7 murine macrophages. BMC Complementary and Alternative Medicine February 2018.18(1)

19. Kyung-Mi Lee, Taejin Park, Min-Seon Kim, Jin-Soo Park. Anti-inflammatory Activities of 7,8-Dihydroxy-4-Methylcoumarin Acetylation Products via NF-κB and MAPK Pathways in LPS-Stimulated RAW 264.7 Cells. Natural Product Communications. .May 2022 17(5):

20. Taewoo Joo, Kandhasamy Sowndhararajan, Sunghyun Hong, Jaehak Lee, Sun-Young Park, Songmun Kim, Jin-Woo Jhoo. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L. Saudi J Biol Sci. 2014 Nov; 21(5): 427–435.

21. Baatartsogt Oyungere, Heekyong Lim. Anti-inflammatory Effects of Magnolia sieboldii Extract in Lipopolysaccharide-Stimulated RAW264.7 Macrophages. Tropical Journal of Pharmaceutical Research December 2013; 12 (6): 913-918 .

22. Chi-Ho Lee2 , Eun-Hee Choi3 , GuanHao Li4 and Kang-Duk Choi1.Measuring the phagocytic activity of cells. Methods in Cell Biology Volume 126, 2015, Pages 287-304.

23. Jae In Jung, Jung Mi Kim, Hee Sung Kim Hyung Seo Kim, and Eun Ji Kim. Immunostimulatory Effect of Wild-Cultivated Ginseng Extract via the Increase in Phagocytosis and Cytokine Secretions in RAW264.7 Macrophages. Journal of the Korean Society of Food Science and Nutrition 2019; 48(6): 686-691

24.XuemeiZhang,YuSong,HuanzhangXiongXinxinCi[a]HongyuLi[a]LuYu[a]LeiZhang[a]XumingDeng,Inhibitory effects of ivermectin on nitric oxide and prostaglandin E_2 production in LPS-stimulated RAW 264.7 macrophages. International Immunopharmacology Volume 9, Issue 3, March 2009, Pages 354-359

25. Kwang SeokAhn[a]Eun JungNoh[b]Kwang-HyunCha[c]Yeong ShikKim[b]Soon SungLim[d]Kuk HyunShin[b]Sang HoonJung .Inhibitory effects of Irigenin from the rhizomes of *Belamcanda chinensis* on nitric oxide and prostaglandin E_2 production in murine macrophage RAW 264.7 cells. Life Sciences. Volume 78, Issue 20, 11 April 2006, Pages 2336-2342

26.AleksandraWojtala,MassimoBonora,Dominika Malinska,PaoloPintonJerzyDuszynski Mariusz R.Wieckowski Methods to Monitor ROS Production by Fluorescence Microscopy and Fluorometry. Methods in Enzymology. Volume 542, 2014, Pages 243-262

27. MauroDegli Esposti .Measuring mitochondrial reactive oxygen species Methods. Volume 26, Issue 4, 2 April 2002, Pages 335-340

28. Jian Lin Yin, Nicholas A Shackel, Amany Zekry, Peter H McGuinness, Craig Richards, Karien Van Der Putten, Geoffrey W McCaughan, Josette M Eris, G Alex Bishop.Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. immunology and cell biology01 June 2001

29.MaureenCronin,MylanPho,DebjaniDutta,JamesC.Stephans,StevenShak,Michael,C.Kiefer Jose M.Esteban ,Joffre B.Baker. Measurement of Gene Expression in Archival Paraffin-Embedded Tissues: Development and Performance of a 92-Gene Reverse Transcriptase-Polymerase Chain Reaction Assay.The American Journal of Pathology, Volume 164, Issue 1, January 2004, Pages 35-42

30. Derk Amsen, Karin E. de Visser & Terrence Town. Approaches to Determine Expression of Inflammatory Cytokines. Inflammation and Cancer pp 107–142

31. ZiqiangWang,WeiJiang,ZhiZhang,MinQian,BingDu,Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. Journal of Ethnopharmacology. Volume144(1): September2012, 145-50

32. Reiko MAEDA, Tomoaki IDA, Hideshi IHARA, Tatsuji SAKAMOTO. Immunostimulatory Activity of Polysaccharides Isolated from *Caulerpa lentillifera* on Macrophage Cells .*Bioscience, Biotechnology, and Biochemistry*, Volume 76, Issue 3, 23 March 2012, Pages 501–505,

33.XiaoDuCheng,QingXiWu,JinZhao,TingSu,YongMingLu,WenNaZhang,YaWang,YanChen. Immunomodulatory effect of a polysaccharide fraction on RAW 264.7 macrophages extracted from the wild *Lactarius deliciosus.*International Journal of Biological MacromoleculesVolume 128, 1 May 2019, Pages 732-739 https://doi.org/10.1016/j.ijbiomac.2019.01.201

34. Changkai Zhou,[1]Jing Gao,[2]Haijun Qu,[1]Long Xu,[1]Bin Zhang,[1]Qie Guo,[1]and Fanbo Jin. Anti-inflammatory Mechanism of Action of Benzoylmesaconine in Lipopolysaccharide-Stimulated RAW264.7 Cells. Evidence-Based Complementary and Alternative Medicine/2022

35. Yue Yang, Ronge Xing, Song Liu. Yukun Qin.Immunostimulatory Effects of Sulfated Chitosans on RAW 264.7 Mouse Macrophages via the Activation of PI3 K/Akt Signaling Pathway. International Journal of Biological Macromolecules 108 November 2017

36.A Castrillo , B de Las Heras, S Hortelano, B Rodriguez, A Villar, L Boscaibition of the nuclear factor kappa B (NF-kappa B) pathway by tetracyclic kaurene diterpenes in macrophages. Specific effects on NF-kappa B-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK. J Biol Chem. . 2001 May 11;276(19):15854-60.