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Production of TNF-α, IL-1β, IL-12 and IFN-γ in murine peritoneal macrophages on treatment with wheat germ agglutinin in vitro: involvement of tyrosine kinase pathways

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Abstract Treatment of macrophages with various doses of wheat germ agglutinin (WGA) for different time intervals resulted in enhanced expression of TNF-α, IL-1β, IL-12 and IFN-γ. The maximum expressions were observed at 24 h with 100 ng/ml of WGA. Enhanced transcription of cytokines TNF-α, IL-1β, IL-12, and IFN-γ was observed at 16 h of WGA treatment by RT-PCR. Pharmacological inhibitor of tyrosine kinase, PI3 kinase, protein kinase C, p42/44, p38, JNK and intracellular calcium immobilizing agent down regulated the WGA induced expression of cytokines TNF-α, IL-1β, IL-12 and IFN-γ. Maximum protein tyrosine kinase activity in macrophages was seen at 5 min of WGA treatment. Maximum cytosolic Ca^{++} was observed at 10 min of WGA treatment. WGA treated macrophages showed maximum activation of protein kinase C (PKC) and PI3 kinase at 10 min, p42/44, p38 at 15 min and JNK at 30 min. Transcription factor ELK1 was activated at 60 min and IêB, c-Fos and c-Jun at 30 min of WGA treatment. The pharmacological inhibitors were also used to check the cascade of activation of tyrosine kinase, PKC, PI3 kinase, p42/44, p38, JNK and release of calcium from intracellular storage to sort out the signal pathways involved in the release of TNF-α, IL-1β, IL-12, and IFN-γ by macrophages on treatment with WGA in vitro.

Keywords WGA · Macrophages · Cytokines · Protein tyrosine kinase

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Introduction

Many macrophage functions involve lectin carbohydrate interaction e.g. adhesion to the endothelium initially relies on the action of selectins (carbohydrate binding proteins) that mediate the attachment of leukocytes to endothelial carbohydrates [1] and recognition and phagocytosis of bacteria in the absence of opsonins most often involve microbial lectins binding to macrophages glycoconjugate receptors, a process called lectinophagocytosis [2]. Plant lectins also possess biological activities and have been used to study variety of biological processes. Many plant lectins are toxic, some may aggregate erythrocytes of different blood groups (hemagglutination), some are associated with colitis, chronic disease celeaic spruce, IBS (intestinal bowel syndrome) and gut permeability [3] and yet others function as mitogens of T and B lymphocytes [4–6]. In relation to neutrophils lectins with different carbohydrate specificities e.g. ConA binding to mannose and WGA binding to *N*-acetyl-D-glucosamine (GlcNAc) and sialic acid have been shown to induce cellular responses [7–10].

WGA was found to stimulate superoxide anion and H_2O_2 production by both paraffin oil and thioglycolate elicited mouse peritoneal macrophages [11–13] and polymorphonuclear cells [14]. WGA induced significant tumoricidal activity in the tumor associated macrophages at different states of activation [15]. Macrophages constitute the major group of phagocytic leukocytes and are crucial to immunosurveillance against invading pathogens and malignancies. Upon activation, macrophages express one or more cytotoxic effector molecule such as peroxidase, cytolytic protease, nitric oxide (NO) and proinflammatory cytokines [16–19]. The production of proinflammatory cytokines by macrophages is one of

the principal effector mechanism and potent inducer of cell cytotoxicity [19–22].

WGA conjugated praecoxin A showed an interesting increase in IL-6 and IL-12 by macrophages [23]. Protein tyrosine phosphorylation is central mechanisms that mediate signal transduction events involved in a wide range of cellular processes. Protein tyrosine kinases activity is often associated with receptor tyrosine kinases (insulin receptor, EGFR, PDGFR, TRKs) and soluble non-receptor tyrosine kinase (p60src, lyn, fyn etc.) [24].

Phosphatidylinositol-4-phosphate-5-kinase catalyze the synthesis of phosphatidylinositol-4,5-bisphosphate, which regulates various processes including cell proliferation, survival, membrane trafficking and cytoskeletal organization. PKCs are protein serine/threonine kinases whose activities are dependent on calcium and phospholipids [25]. The MAP kinases are proline directed serine/threonine kinase and consist of three subfamilies identified as the extra cellular signal regulated kinase or ERK which include p42 (ERK1) and p44 (ERK2), c-Jun amino terminal kinase or stress activated protein kinase (JNK/SAPK) and p-38 subgroup of kinase [26, 27]. WGA induces rapid tyrosine phosphorylation [28] and phospholipase C gamma activation by Src family kinase [29] in platelets and spermatozoa. In the present study we have observed signaling pathways involved in the production of TNF-α, IL-1β, IL-12 and IFN-γ from murine peritoneal macrophages on treatment with wheat germ agglutinin in vitro.

Materials and methods

Mice

Inbred strains of BALB/c mice of either sex at 8–10 weeks of age were used for obtaining peritoneal macrophages.

Cell cultures and reagents

Macrophage cell cultures were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamycin (20 ug/ml) at 37°C in humidified air containing 5% CO_2. Medium RPMI 1640, TRI-reagent, lipopolysaccharide (LPS), wheat germ agglutinin (from *Triticum vulgaris*), TMB-8 hydrochloride, wortmannin, Fura-2AM, protein tyrosine kinase kit, *N*-acetyl-D-glucosamine (GlcNAc) and most of the other reagents were obtained from Sigma–Aldrich Chemicals, St. Louis, Missouri, USA. Fetal calf serum was purchased from Biological Industries, Haemaek, Israel. Genestein was from LC Services Inc., USA. PD98059, SB202190, SP600125 and H-7 were purchased from Calbiochem, La Jolla, CA, USA. Polyclonal antibodies against phospho-PI3K, PKC, phospho-p42/44, phospho-p38, phospho-JNK, phospho-ELK1, phospho-IκB, c-Fos, phospho-cJun, actin and HRP-conjugated anti-mouse, anti-rabbit and anti-goat IgGs were obtained from Santa Cruz Biotechnology Inc, Santa Cruz, California, USA. One-step

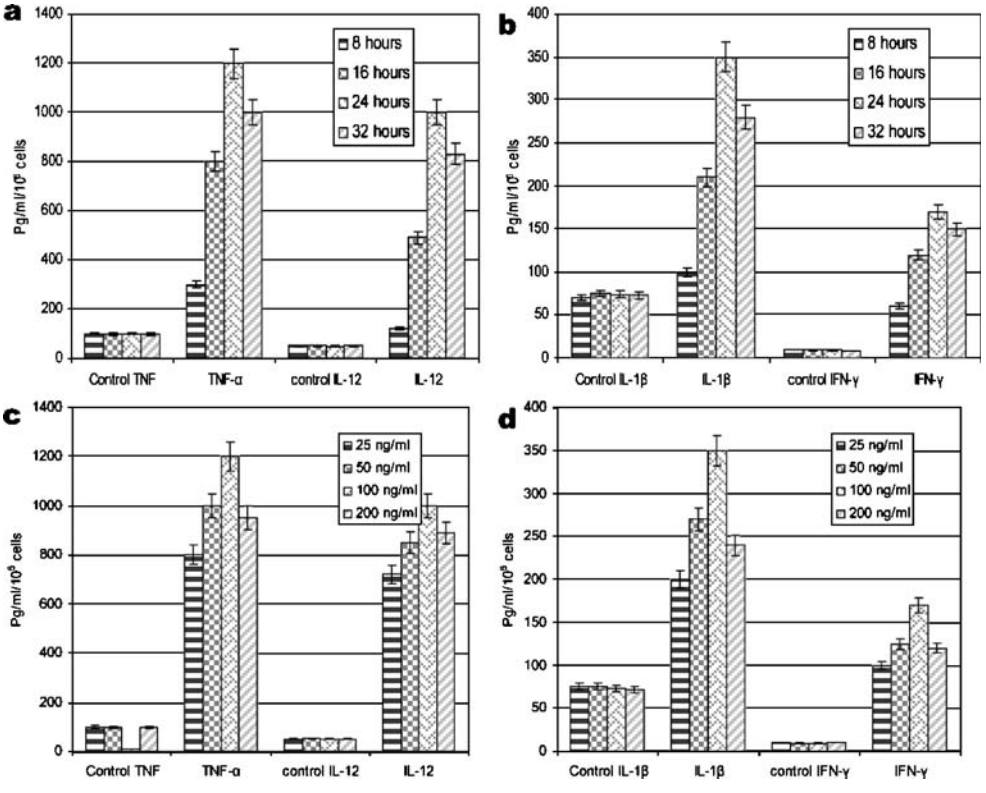

Fig. 1 **a**–**d** Time and dose kinetics of TNF-α, IL-1β, IL-12 and IFN-γ production by macrophages treated with WGA as measured by ELISA. All values are mean ± SD and representative of three independent experiments. $P<0.05$, of WGA treated macrophages was significantly different from respective control

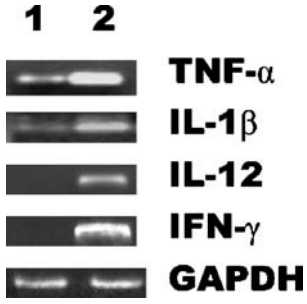

Fig. 2 mRNA expression of TNF-α, IL-1β, IL-12 and IFN-γ in WGA treated macrophages by RT-PCR. *Lane-1*, untreated macrophages; *Lane-2*, WGA treated macrophages for 16 h. The *lowest panel* represents the expression of GAPDH. The figure is representative of three independent experiments with similar result

RT-PCR kit was from Qiagen, Germany. Mouse primers for TNF-α, IL-1β, IL-12, IFN-γ and GAPDH were purchased from GENSET Singapore Biotech. Pvt. Ltd, Singapore. All the reagents were endotoxin-free as determined by the *Limulus* amoebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

Isolation and activation of macrophages

Macrophage monolayers (10^6 cells/well) were prepared as described previously [30]. Peritoneal exudate cells were harvested from peritoneal lavage using chilled serum-free RPMI 1640 medium and added to wells of 24 well tissue culture plates (Nunc, Denmark). After 2 h incubation at 37°C in an atmosphere of 5% CO_2 in air in a CO_2 incubator, the non-adherent cells were removed by vigorous washing (three times) with warm serum-free medium and the adherent cells were further incubated in complete medium overnight to form macrophage monolayers. More than 95% of the adherent cell population was macrophages as determined by morphology and non-specific esterase staining. Macrophage monolayers were treated with wheat germ agglutinin (100 ng/ml) for different time intervals in fresh medium. In another set, the macrophage monolayers were pretreated with optimum doses of protein tyrosine kinase inhibitor, genestein (10 μg/ml) or PI3 kinase inhibitor, wortmannin (200 nm) or an intracellular calcium immobilizing agent, TMB-8 (100 μM) or p42/44 inhibitor, PD98059 (10 μM) or p38 inhibitor, SB202190 (10 μM) or protein kinase C inhibitor, H-7, (10 μM) or JNK inhibitor, SP600125 (10 μM) for 1 h and were further incubated with 100 ng/ml of wheat germ agglutinin in fresh medium for different time periods [31–33]. The strength of DMSO in the stock solutions of above inhibitors was adjusted so that its effective concentration was less than 0.1% when used at the recommended doses.

RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from the macrophages by TRI-reagent in accordance with the supplier's instructions. The RNA was reverse-transcribed using a one-step RT-PCR kit

Table 1 Effect of different doses of different pharmacological inhibitors on WGA induced TNF-α, IL-1β, IL-12, IFN-γ production and viability after 1 h of inhibitors treatment in macrophages

Treatment	TNF-α (pg)/ 10^6cells/ml		IL-1β(pg)/ 10^6cells/ml		IL-12(pg)/ 10^6cells/ml		IFN-γ(pg)/ 10^6cells/ml		%viability of cells after 1 hr of inhibitor treatment
	Medium	WGA	Medium	WGA	Medium	WGA	Medium	WGA	
Medium	100±3.2	1200±4.4	75±5.6	350±6.7	50±6.7	1000±11.2	10±.7	170±4.8	100%
Genestein (8 μg/ml)	95±3.5	750±5.5	72±7.5	210±10.2	48±7.3	620±10.6	9±.6	120±4.4	99%
(10 μg/ml)	97±4.0	600±7.1	73±6.9	170±9.7	46±7.8	490±4.9	8±1.2	80±5.4	99%
(12 μg/ml)	85±3.9	450±7.5	65±6.2	120±6.5	41±7.2	350±5.5	6±.4	50±6.9	84%
Wortmannin (150 nM)	99±5.1	810±6.1	74±6.7	220±9.5	47±4.6	690±6.7	8±1.0	140±2.1	98%
(200 nM)	96±5.8	630±6.7	74±6.8	180±6.8	48±7.3	490±5.5	9±.3	120±8.0	98%
(250 nM)	91±4.7	510±6.6	67±5.4	150±7.2	38±7.8	380±11.2	6±1.1	90±4.6	87%
H-7 (8 μM)	99±6.2	820±6.0	74±4.5	250±6.9	49±3.4	710±7.2	8±.6	140±6.7	99.2%
(10 μM)	97±5.1	710±6.1	73±4.9	200±7.1	48±7.2	590±4.1	9±.7	95±10.2	99%
(12 μM)	91±5.8	530±6.2	66±6.8	170±6.4	36±6.7	430±4.9	7±1.2	60±4.2	82%
TMB-8 (80 μM)	100±5.4	810±5.4	75±3.2	240±9.2	49±3.2	750±2.1	9±1.4	120±5.1	99.4%
(100 μM)	99±5.3	730±5.5	74±3.9	220±10.5	49±7.4	600±4.2	9±.9	100±7.8	99.2%
(120 μM)	89±5.2	550±5.6	65±5.5	160±3.2	42±7.4	410±8.1	6±.3	70±6.2	92%

All values are mean±SD and representative of three independent experiments. Values of medium (alone) or inhibitors (alone) are statistically different from WGA treated sample alone or with inhibitors used as indicated. All values are significant with $P<0.05$.

Table 2 Effect of different doses of different pharmacological inhibitors and *N*-acetyl-D-glucosamine on WGA induced TNF-α, IL-1β, IL-12, IFN-γ production and viability after 1 h of inhibitors treatment in macrophages

Treatment	TNF-α (pg)/ 10^6cells/ml		IL-1β(pg)/ 10^6cells/ml		IL-12(pg)/ 10^6cells/ml		IFN-γ(pg)/ 10^6cells/ml		%viability of cells after 1 hr of inhibitor treatment
	Medium	WGA	Medium	WGA	Medium	WGA	Medium	WGA	
Medium	100±3.2	1200±4.4	75±5.6	350±6.7	50±6.7	1000±11.2	10±.7	170±4.8	100%
PD98059 (8 μM)	96±5.7	850±7.9	74±7.7	300±6.3	47±7.1	810±4.6	9±.6	130±7.8	99.2%
(10 μM)	98±5.2	790±8.1	73±6.8	270±4.9	47±6.2	730±8.2	9±.8	110±5.9	99%
(12 μM)	88±5.1	650±8.2	63±6.7	200±8.9	38±8.9	610±4.5	6±.7	80±7.8	93%
SB202190 (8 μM)	97±3.8	880±5.9	75±7.7	310±8.2	49±9.5	840±6.3	8±.8	140±5.4	99.5%
(10 μM)	99±3.6	820±5.6	74±8.9	280±7.6	48±6.2	710±12.3	8±1.9	130±10.6	99.4%
(12 μM)	86±3.8	670±5.8	62±8.8	190±4.8	37±7.8	630±5.7	6±.8	100±7.3	93.6%
SP600125 (8 μM)	98±4.3	930±6.7	75±6.9	300±3.7	48±9.2	850±7.6	9±.1	150±6.6	99.3%
(10 μM)	98±4.1	870±6.9	74±7.8	290±3.6	47±3.5	730±6.7	8±.5	140±6.8	99.2%
(12 μM)	87±4.4	700±7.2	67±7.4	200±8.8	43±7.8	650±5.6	6±.9	110±8.2	94%
GlcNAc (10 mM)	90±3.7	920±5.8	71±6.2	260±5.6	44±6.9	830±6.4	9±.7	140±7.2	98.5%
GlcNAc (25 mM)	94±4.2	710±4.9	73±5.6	220±6.4	42±5.3	690±7.2	8±.6	90±6.4	98.2%
GlcNAc (50 mM)	90±4.5	610±4.2	68±6.2	190±5.9	40±3.2	570±3.4	7±.5	70±4.7	95.1%

All values are mean±SD and representative of three independent experiments. Values of medium (alone) or inhibitors (alone) are statistically different from WGA treated sample alone or with inhibitors used as indicated. All values are significant with $P<0.05$.

and amplified by PCR using the specific murine primers indicated in the 'Results'. The thermo cycle conditions were 28 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, after which an additional extension step at 72°C for 10 min was included. Electrophoresis of amplified DNAs was carried out on a 2% agarose gel and stained with ethidium bromide. The murine primer sequences are as follows:

TNF-α forward 5′-GGAGGTCTACTTTGGAGTCATTGC-3′
TNF-α reverse 5′-TCCCTTTGCAGAACTCAGGAATGG-3′
IL-1β forward 5′-CATGGGATGATGATGATAACCTGCT-3′
IL-1 β reverse 5′-CCCATACTTTAGGAAGACAGGGATTT-3′
IL-12 forward 5′-CCACTCACATCTGCTGCTCAACAAG-3′
IL-12 reverse 5′-GATGCCAAAGTTGTCATGGATGTCC-3′
IFN-γ forward 5′-GGTGACATGAAAATCCTGCAGAGC-3′
IFN-γ reverse 5′-CGCTGGACCTGTGGGTTGTTGACC-3′
GAPDH forward 5′-CCTGCAGTGTCTGATATTGTTG-3′
GAPDH reverse 5′-AACACACCATTGCGATGAA-3′.

To show the equal loading of RNA same volume of same sample was taken and expression of housekeeping gene GAPDH was checked. The possible contamination of any PCR component was excluded by performing a PCR reaction with these components in the absence of RT product in each set of experiment (negative control—data not shown).

Preparation of cell lysates and immunoblotting

The macrophage monolayers with or without treatment with wheat germ agglutinin were washed with ice cold phosphate buffered saline containing 1 mM Na_3VO_4, then lysed in 50 μl of lysis buffer [20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin and 0.15 units/ml aprotonin] for 20 min at 4°C. The lysates were centrifuged at 10,000×*g* for 10 min and the supernatants (containing Triton X-100 soluble proteins) were separated on 10% SDS-polyacrylamide gels at 20 mA. The separated proteins were transferred to nitrocellulose membrane (1 h at 125 V using Bio-Rad mini trans blotter), and immunoblotted with primary antibody, incubated with horseradish peroxidase conjugated secondary antibody and visualized by the chemiluminescence Western blotting kit (Santa Cruz Biotechnology, California, USA). To show the equal loading of protein, same volume of same sample was taken and blot was developed for actin.

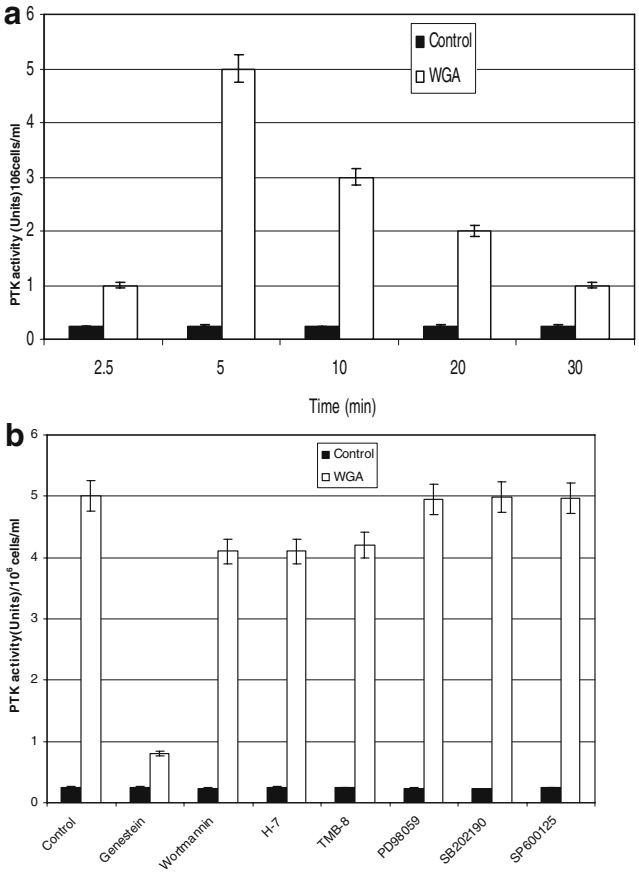

Fig. 3 **a** Time kinetics of PTK activity in macrophages treated with WGA (100 ng/ml). Each *bar* represents the standard error of three independent experiments. All values are mean±SD and representative of three independent experiments. $P<0.05$, of WGA treated macrophages was significantly different from respective control. **b** Effect of Inhibitors on PTK activity on macrophages after 5 min of WGA (1 ng/ml) treatment. Each *bar* represents the standard error of three independent experiments. All values are mean ± SD and representative of three independent experiments. $P<0.05$, of WGA treated macrophages was significantly different from respective control

Protein tyrosine kinase (PTK) assay

PTK activity was measured using colorimetric kit. Briefly, cell lysate was prepared using lysis buffer containing activated sodium vanadate solution (according to kit instruction). Assay was performed in a 96-well microtiter plate. One hundred twenty-five microliters of PTK substrate solution was added to each well and plate was incubated overnight at 37°C. Coating solution was removed and each well was washed with 200 μl of washing buffer. Buffer was removed and wells were dried for 2 h at 37°C. Ninety microliters of 1X tyrosine kinase buffer containing ATP was added to each well. Twenty microliters of cell lysates was added in each well. Plate was covered and incubated at room temperature for 30 min. Reaction mixture was removed and each well was washed with 200 μl of washing buffer five times. In each well 100 μl conjugated antibody was added. Plate was

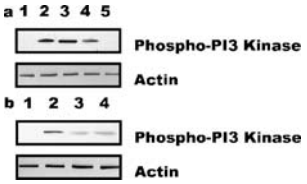

Fig. 4 **a** Time kinetics of phospho-PI3K in WGA (100 ng/ml) treated macrophages by immunoblotting. *Lane-1*, untreated macrophages; *Lane-2*, WGA treated macrophages for 5 min; *Lane-3*, WGA for 10 min; *Lane-4*, WGA for 15 min; *Lane-5*, WGA for 30 min. The *lower panel* represents the expression of actin. The figure is representative of three independent experiments with similar result. **b** Effect of genestein and wortmannin on the expression of phospho-PI3K in WGA treated macrophages by immunoblotting. *Lane-1*, untreated macrophages; *Lane-2*, WGA (100 ng/ml) treated macrophages for 10 min; *Lane-3*, WGA + genestein for 10 min; *Lane-4*, WGA + wortmannin for 10 min. The *lower panel* represents the expression of actin. The figure is representative of three independent

covered and incubated at room temperature for 30 min. Antibody solution was removed and each well was washed with 200 μl of washing buffer five times. One hundred microliters of freshly prepared OPD substrate solution was added to each well and incubated for 7 min in dark at room temperature. One hundred microliters of 2.5N H_2SO_4 was added to each well to stop the reaction. Plate was read in a microplate ELISA reader (Emax, Molecular Devices, USA) at 492 nm within 30 min of addition of stop solution.

Cytosolic and membrane fractionation for assaying the PKC activation

Macophages (1×10^6 cells/ml) were lysed in ice-cold buffer containing 20 mM HEPES (pH 7.5), 2 mM EDTA, and 10 mM EGTA. Membrane fractions were obtained after cen-

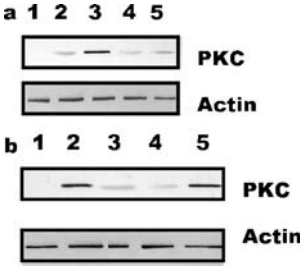

Fig. 5 **a** Time kinetics of PKC activation in WGA (100 ng/ml) treated macrophages by immunoblotting in membrane fraction. *Lane-1*, untreated macrophages; *Lane-2*, WGA treated macrophages for 5 min; *Lane-3*, WGA for 10 min; *Lane-4*, WGA for 15 min; *Lane-5*, WGA for 30 min. The *lower panel* represents the expression of actin. The figure is representative of three independent experiments with similar result. **b** Effect of genestein, H-7 and wortmannin on the expression of PKC activation in WGA treated macrophages by immunoblotting membrane fraction. *Lane-1*, untreated macrophages; *Lane-2*, WGA (100 ng/ml) treated macrophages for 10 min; *Lane-3*, WGA + genestein for 10 min; *Lane-4*, WGA + H-7 for 10 min; *Lane-5*, WGA + wortmannin for 10 min. The *lower panel* represents the expression of actin. The figure is representative of three independent experiments with similar result

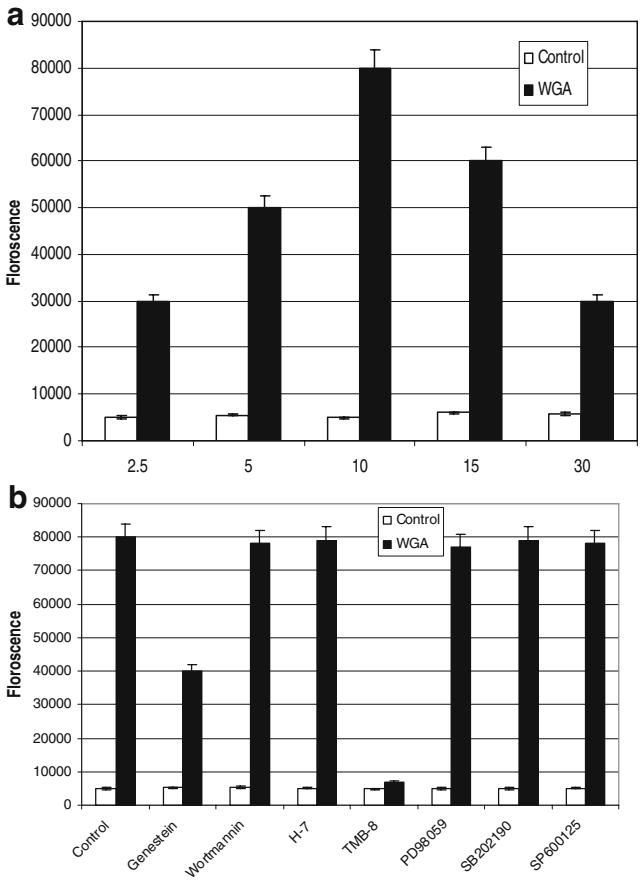

Fig. 6 **a** Time kinetics of Ca^{++} release from intracellular storage into cytoplasm of macrophages treated with WGA (100 ng/ml) as measured by Fura-2AM staining. Each *bar* represents the standard error of three independent experiments. All values are mean ± SD and representative of three independent experiments. $P<0.05$, of WGA treated macrophages was significantly different from respective control. **b** Effect of Inhibitors on Ca^{++} release in macrophages after 10 min of WGA (100 ng/ml) treatment as measured by Fura-2A staining. Each *bar* represents the standard error of three independent experiments. All values are mean ± SD and representative of three independent experiments. $P<0.05$, of WGA treated macrophages was significantly different from respective control

trifugation at 20,000×*g* for 1 h. The supernatant fluid contained the cytosolic fraction enzyme. The membrane fraction was extracted from the pellet after 0.1% Triton X-100 treatment and centrifugation at 20,000×*g* for 45 min. PKC activity was checked in membrane fraction by immunoblotting.

Ca^{++} measurement in macrophage by Fura-2AM

Macrophage monolayers were treated with WGA (100 ng/ml) for different time intervals. In another set macrophage monolayers were pretreated with different pharmacological inhibitors for 1 h followed by treatment with WGA in a 96 well tissue culture plate for different time intervals. After that complete medium was discarded and the cells were washed with warm incomplete medium. Five hundred microliters of fresh complete medium was added. Then 1 μl of Fura-2AM (2 μM) was added and incubated for 30 min at 37°C. Medium was then removed and a fresh complete medium was added and kept for 30 min at 37°C. After 30 min, cells were rinsed three times with 500 μl of KRH solution (NaCl—125 mM, KCl—5 mM, $MgSO_4$—1 mM, Glucose—6 mM, $CaCl_2$—2 mM, HEPES—10 mM, pH—7.4). Plate was read in plate spectrofluorometer (FLx 800 Microplate Fluorescence Reader, BIOTEK Instruments Inc. USA) with an excitation filter 340/30 nm and an emission filter 508/20 nm.

Assay for TNF-α, IL-1β, IL-12 and IFN-γ

Macrophage monolayer was treated with different doses of WGA for different time intervals. In another set macrophages monolayers were pretreated with different pharmacological inhibitors for 1 h washed and treated with WGA for 24 h or coincubated with WGA and GlcNAc for 24 h. TNF-α, IL-1β, IL-12 and IFN-γ were measured in supernatant by commercial ELISA kits from BD Pharmingen, California, USA.

Percentage viability by MTT Assay

Percentage viability of macrophages was determined by MTT (3-(4,5)-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay as described earlier [34]. The relative cell viability was calculated according to the formula:

$$\text{Relative cell viability} = \frac{\text{Absorbance Experimental}}{\text{Absorbance Control}} \times 100$$

Where, 'Absorbance Control' represents macrophages incubated in medium alone and 'Absorbance Experimental' represents macrophages treated with the lectin or inhibitor or their vehicles. In each of the experiments, the viability of the peritoneal macrophages was not affected by the doses of the lectin or inhibitor or their vehicles used up to 48 h.

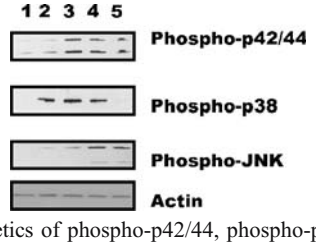

Fig. 7 Time kinetics of phospho-p42/44, phospho-p38 and phospho-JNK protein in WGA (100 ng/ml) treated macrophages by immunoblotting. *Lane-1*, untreated macrophages; *Lane-2*, WGA treated macrophages for 5 min; *Lane-3*, WGA for 15 min; *Lane-4*, WGA for 30 min; *Lane-5*, WGA for 60 min. The *lowest panel* represents the expression of actin of same sample using same volume as with different MAP kinases. The figure is representative of three independent experiments with similar result

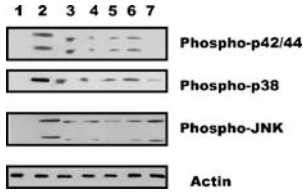

Fig. 8 Effect of genestein, wortmannin, H-7, TMB-8, PD98059 (phospho-p42/44) or SB202190 (phospho-p38) or SP600125 (phospho-JNK) on the expression of phospho-p42/44, phospho-p38 and phospho-JNK protein in WGA treated macrophages by immunoblotting. *Lane-1*, untreated macrophages; *Lane-2*, WGA (100 ng/ml) treated macrophages for 15/30 min; *Lane-3*, WGA + genestein for 15/30 min; *Lane-4*, WGA + wortmannin for 15/30 min; *Lane-5*, WGA + H-7 for 15/30 min; *Lane-6*, WGA + TMB-8 for 15/30 min; *Lane-7*, WGA + PD98059 or SB202190 or SP600125 for 15/30 min. The *lowest panel* represents the expression of actin of same sample using same volume as with MAP kinases. The figure is representative of three independent experiments with similar result

Statistical analysis

The statistical significance of difference between the test groups was analyzed by ANOVA.

Results

1. *WGA induced TNF-α, IL-1β, IL-12 and IFN-γ production by macrophages.*

Treatment of macrophages at different time intervals with different doses of WGA resulted in the enhanced production of TNF-α, IL-1β, IL-12 and IFN-γ. Maximum production was observed with 100 ng/ml of WGA at 24 h (Fig. 1a–d). Viability of macrophages after 24 h at 100 ng/ml was the same as untreated cells (data not shown).

Macrophages treated with WGA (100 ng/ml) showed significantly increased transcription of TNF-α, IL-1β, IL-12 and IFN-γ at 16 h (Fig. 2).

2. *Signaling molecules involved in TNF-α, IL-1β, IL-12 and IFN-γ production by WGA activated macrophages.*

Pretreatment of macrophages with genestein or wortmannin or H-7 or TMB-8 or PD98059 or SB202190 or SP600125 for 1 h with different doses followed by treatment with WGA for 24 h resulted in the inhibition of TNF-α, IL-1β, IL-12 and IFN-γ production by macrophages. Macrophages incubated with inhibitor alone did not have any effect on cytokine production (Tables 1 and 2).

3. *PTK activity in WGA treated macrophages*.

PTK activity was measured in macrophages treated with WGA (100 ng/ml) for various time intervals. Maximum activity was observed at 5 min (Fig. 3a). Treatment of macrophages with pharmacological inhibitors genestein inhibited the PTK activity. Wortmannin, H-7, and TMB-8 also resulted in reduced PTK activity, while PD98059, SB202190 and SP600125 did not have any effect (Fig. 3b).

4. *WGA activates PI3K and PKC in time dependent manner.*

WGA treatment of macrophages resulted in the activation of *PI3K* and PKC. Maximum activation of PI3K and PKC was observed at 10 min of incubation (Figs. 4a and 5a). Genestein and wortmannin down regulated the expression PI3K. PKC expression was inhibited by genestein and H-7. Wortmannin did not have any effect on PKC activation (Figs. 4b and 5b).

5. *WGA activates Ca^{++} release from intracellular storage*.

WGA treatment of macrophages resulted in the release of Ca^{++} from intracellular storage. Maximum cytosolic Ca^{++} release was observed at 10 min of incubation with WGA (Fig. 6a). Genestein and TMB-8 inhibited the release of Ca^{++} while wortmannin, H-7, SP600125, SB202190 and PD98059 did not have any effect on Ca^{++} release (Fig. 6b).

6. *WGA activates MAP Kinase p42/44, p38 and JNK in a time dependent manner.*

Treatment of macrophages with WGA (100 ng/ml) for various time intervals resulted in the expression of phospho-p42/44, phospho-p38 and phospho-JNK. The maximum activation (phosphorylation) of p42/44 and p38 was observed at 15 min and JNK at 30 min (Fig. 7).

7. *Pharmacological inhibitor of upstream signaling molecules blocks the activation of down stream signaling molecules*.

Pretreatment of macrophages with genestein or wortmannin or H-7 or TMB-8 or PD98059 or SB202190 or SP600125 for 1 h, followed by 15 min or 30 min treatment of WGA resulted in down regulation of the expression of phospho-p42/44, phospho-p38 and phospho-JNK (Fig. 8).

8. *WGA activates transcription factors Elk1, NF-kB, c-Fos, c-Jun in time dependent manner.*

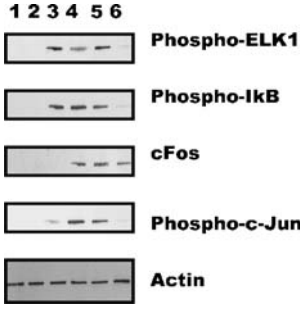

Fig. 9 Time kinetics of phospho-ELK1, phospho-IκB, c-Fos and phospho-c-Jun protein in WGA (100 ng/ml) treated macrophages by immunoblotting. *Lane-1*, untreated macrophages; *Lane-2*, WGA treated macrophages for 5 min; *Lane-3*, WGA for 15 min; *Lane-4*, WGA for 30 min; *Lane-5*, WGA for 60 min; *Lane-6*, WGA for 120 min. The *lowest panel* represents the expression of actin of same sample using same volume as with different transcription factors. The figure is representative of three independent experiments with similar result

Treatment of macrophages with WGA resulted in activation of transcription factors with maximum activity at different time intervals. Phospho-Elk1 showed maximum expression at 60 min. The maximum expression of phospho-IkB, c-Fos and phospho-c-Jun was seen at 30 min (Fig. 9).

9. *N-acteyl–glucosamime (GlcNAc) inhibits WGA induced cytokine production in macrophages.*

Coincubation of macrophages with WGA in presence of GlcNAc resulted in reduced expression of cytokines TNF-α, IL-1β, IL-12 and IFN-γ (Table 2).

Discussion

In the present study it is reported that WGA induces the activation of macrophages in vitro, as manifested by the production of proinflammatory cytokines TNF-α, IL-1β, IL-12 and IFN-γ. Secretion of cytokines by activated macrophages is central to their immunoregulatory role and the orchestration of a robust immune response by macrophages. Macrophages maintain an effective immune response at the site of inflammation and malignancy [35]. TNF-α, IL-1β, IL-12 and IFN-γ production by the macrophages in response to WGA was found to be dose and time dependent that was sensitive to different pharmacological inhibitors. The optimum time for their gene transcription was 16 h with 100 ng/ml of WGA. The maximum production of TNF-α, IL-1β, IL-12 and IFN-γ by WGA treated macrophages as measured by ELISA was at 24 h with 100 ng/ml. Joo et al. [23] have reported that WGA conjugated praecoxin A induces increase in IL-6 and IL-12 secretion by macrophages. This is probably the first report showing the direct effect of WGA on macrophages.

The receptor on cell surface after ligand binding triggers a cascade of events involving protein phosphorylation, calcium flux and generation of membrane inositol derived secondary messenger culminating in the transcription of proinflammatory cytokine genes like IL-1β and TNF-α [36–41]. Protein phosphorylation mediated by tyrosine kinase and serine/threonine kinases have been correlated with the production of TNF-α and other cytokines in macrophages activated by agents such as LPS, interleukins and the anticancer drug cisplatin [3, 41]. However, the pathways involved in macrophage activation by WGA have not been investigated. We, therefore, investigated the role of protein tyrosine kinase, serine/threonine kinase and other signaling molecules in the WGA induced cytokines production in murine peritoneal macrophages. For this, tyrosine kinase inhibitors—genestein, PI3 kinase inhibitor—wortmannin, PKC inhibitor—H-7, an intracellular Ca^{++} immobilizing agent—TMB-8, p42/44 inhibitor—PD98059, p38 inhibitor—SB202190, JNK inhibitor—SP600125 were used. It is observed that WGA induced TNF-α, IL-1β, IL-12 and IFN-γ production was sensitive to genestein, wortmannin, H-7, TMB-8, PD98059, SB202190 and SP600125 in a dose dependent manner. This suggests that protein tyrosine kinase, PI3 kinase, PKC, Ca^{++}, p42/44 MAPK, p38 MAPK and JNK MAPK pathways are involved in WGA induced signal transduction of macrophage activation.

The critical involvement of protein tyrosine kinases in signal transduction pathways is well established. Ligand-induced tyrosine phosphorylation of such receptor induces receptor dimerization and subsequent autophosphorylation of specific individual phosphotyrosine residues located within their cytoplasmic domains, which serve as binding sites that interact with specific cytoplasmic molecules [42–44]. MAP kinases play crucial role in many aspects of immune mediated inflammatory responses. Hence, member of this family of kinases have come to be appreciated as key cellular signal transducers and attractive target for drug development [45, 46]. To further confirm our observations we checked the direct involvement of tyrosine kinase, PKC, Ca^{++}, PI3 kinase and MAP kinases. PTK activity was maximum at 5 min after WGA treatment. Maximum activation of PI3K, PKC and Ca^{++} release was found at 10 min. Maximum activation of p42/44 and p38 was observed at 15 min and JNK at 30 min. By using different pharmacological inhibitors, it is observed that PTK activity was inhibited maximally by genestein and partially by H-7, TMB-8 and wortmannin, but PD98059, SB202190, SP600125 did not have any effect on PTK activity. Genestein and wortmannin inhibited the expression of PI3K. Wortmannin did not have any effect on PKC expression but genestein and H-7 inhibited the PKC expression. Calcium release was inhibited by genestein and TMB-8, while wortmannin, H-7, PD98059, SB202190 and SP600125 did not have any effect. It is observed that activation of p42/44, p38 and JNK was sensitive to genestein, wortmannin, H-7, TMB-8 and PD98059 or SB202190 or SP600125. Transcription factor Elk1 was activated maximally at 60 min while NF-κB (as observed by Western blotting for phospho-IκB), c-Fos, c-Jun at 30 min of WGA treatment. The inhibition of WGA induced cytokine production by GlcNAc suggests the involvement of cell surface *N*-acetyl-D-glucosamine. However, the role of other sugar moieties in WGA induced cytokine production cannot be ruled out. This could be the reason that GlcNAc inhibits WGA induced cytokine production significantly, but does not abrogate it completely.

Conclusion

Based on the data with pharmacological inhibitors, it is suggested that receptor for WGA is tyrosine kinase associated. PI3K and PKC are activated by tyrosine kinases. Wortmannin

did not have any effect on PKC activation suggested that PI3K and PKC follow different pathways. Calcium signal was blocked by genestein but not by wortmannin and H-7. This confirms that upstream tyrosine kinases are shared by all the molecules, after that they follow separate downstream pathways. Finally all pathways may converge at MAP kinases, since the expressions of all three MAP kinases were inhibited by genestein, wortmannin, TMB-8 and H-7. Finally MAP Kinases activate different transcription factors. These transcription factors then participate in the activation of macrophages for release of inflammatory cytokines like TNF-α, IL-12, IFN-γ and IL-1β. Identification of different signaling molecules involved in WGA mediated macrophage activation will help in the understanding of physioimmunology involved in different inflammatory disorders and may be utilized pharmacologically for the treatment of these disorders.

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