Research Article

Mannose-rich guar gum nanoparticles as a novel therapeutic drug against inflammatory diseases

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Received on March 24, 2018; Accepted on May 29, 2018; Published on May 31, 2018

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Abstract

The potential to deliver nanoparticles, like polymer-based nanoparticles that can be enriched with functional groups to ensure entry into cells, directly into targeted cells is important for the therapy of inflammatory diseases. Plant-derived nanoparticles, with inherent anti-inflammatory activity and modified to allow receptor-mediated uptake, can be used as effective therapy with minimal side effects. The particle used in this study is an edible polysaccharide, derived from *Cyamopsis tetragonoloba*, with a galactomannan component. The particle was made mannose-rich to increase specificity towards cells expressing mannose receptors, and initially tagged with rhodamine isothiocyanate to trace its path. This study aimed to determine the therapeutic effect of the guar gum nanoparticle (GN) *in vitro* and *in vivo* in inflammatory diseases. *In vitro* studies on RAW 264.7 cells showed successful uptake of the nanoparticle, in a short duration of time, via their mannose receptors. Nitric oxide and MTS assays showed anti-inflammatory effects of GN. *In vivo* mouse model of thioglycollate-induced peritonitis showed significant decrease in inflammation, indicating its anti-inflammatory effect, and increase in clonogenic potential, indicating its regenerative potential, on intraperitoneal administration of GN. The results reflect the potential of the nanoparticle in cellular trafficking, site- specific drug delivery and bioimaging applications.

Introduction

Guar gum (GG), also known as guar flour, is a non-ionic polysaccharide gum, obtained from the ground endosperm of the seed of the leguminous guar plant, *Cyamopsis tetragonoloba* (Sahoo *et al.* 2013). It is an almost odourless and tasteless powder, with a whitish yellow colour. The guar beans have an endosperm containing a galactomannan gum (Saikh and Kumar, 2011), made of high molecular weight polysaccharides (Sahoo *et al.* 2013). The guar powder is insoluble in organic solvents, but forms a gel-like consistency in water (Sahoo *et al.* 2013). The GG polysaccharide comprises 80% galactomannan, being made of (1→4)-β-D mannopyranosyl (mannose) linear chains attached to units of α-D-galactopyranose, by (1→6) glycosidic linkages (Hartemink *et al.* 1999, Sahoo *et al.* 2013). Mannose and galactose are usually present in a ratio of 2:1 (Hartemink *et al.* 1999, Sahoo *et al.* 2013, Saikh & Kumar 2011). GG is used in various fields, like in the food industry (Hartemink *et al.* 1999, Sahoo *et al.* 2013), and in the pharmaceutical industry (Saikh & Kumar 2011). Guar gum has been used as a drug delivery system in the form of tablets and capsules, allowing for the slow release of the drug (Sahoo *et al.* 2013).

In recent years, a wide variety of different nanoparticles have been synthesized with different surface chemical properties. The guar gum nanoparticle (GN), used in this study, has been designed for uptake in cell systems (Ghosh *et al.* 2015). The GN, prepared by acid hydrolysis of GG, has been extracted without the use of any organic solvent, modified covalently and then made to undergo an epichlorohydrin reaction in liquid ammonia (Ghosh *et al.* 2015). The resultant GN molecule was spherical in shape and has a size of about 80 nm, with a mannose: galactose ratio of 4:1. The GN molecules were further covalently functionalized in aqueous environment with rhodamine isothiocyanate (RITC) using a hydroxypropyl amine spacer group, for potential applications in bioimaging (Ghosh *et al.* 2015).

The ability to be taken up by cells depend upon the nanoparticle's size, its surface-to-charge ratio, and its ability to bind to cell membranes. Nanopar-

ticles show high affinity for cell membranes due to electrostatic interactions, which are mainly decided by the zeta potential of the nanoparticles (Honery & Zahir 2013).

Macrophages, which have mannose receptors on their surfaces, play a critical role in the innate and adaptive immune responses. The mannose rich GN was used in this study to check its uptake via mannose receptors expressed on different cells, like macrophages. The mannose receptor (MR), a C-type lectin that helps in internalization of endogenous, as well as exogenous microbe-derived, substances, recognizes carbohydrate molecules by its cysteine-rich domains (Leteux *et al.* 2000, Liu *et al.* 2000, McKenzie *et al.* 2007). Macrophages also express galactose receptors. Macrophage galactose binding lectin (MGL) is also a C-type lectin on macrophages, which bind to galactose and related sugars (Kawasaki *et al.* 1986, Suzuki *et al.* 1996) and is a very efficient receptor for internalization (Higashi *et al.* 2002).

Peritonitis is the inflammation of the peritoneum, the thin covering that lines the inner wall of the abdomen, covering the organs present in the abdominal cavity (Mitra *et al.* 2015). Peritonitis is caused by disruptions of the peritoneum or due to some systemic infection (Mitra *et al.* 2015). Peritonitis, induced in mice using thioglycollate (TG) leads to an increase in peritoneal macrophages within 24 hours of infection (Lam *et al.* 2013, Zhang *et al.* 2008).

The objective of this work can be divided into two main parts: the *in vitro* studies on murine macrophage cell lines and the *in vivo* studies in a mouse model of an inflammatory disease, i.e. aseptic peritonitis. The *in vitro* studies were done to determine the time over which GN was taken up by the cells, to assess whether the GN was taken up by the cells via their mannose receptors, and to assess whether GN has any anti-inflammatory and/ or proliferative effect on the cells. The *in vivo* studies were done to assess the anti-inflammatory effects of GN in TG induced peritonitis, where the involvement of macrophages, containing mannose receptors, has been documented.

Table 1. Percentage uptake of GN by RAW 264.7 cells with time. RAW 264.7 cells were incubated with GN for 30 and 60 mins. Uptake increased from 70.3% in 30 mins to 96.9% in 60 mins.

Time	Total no. of cells	GN^+ cells	Percentage GN uptake
30 min	37.0 ± 5.0	26.0 ± 6.0	70.3%
60 min	33.0 ± 3.5	32.0 ± 3.5	96.9%

Materials and Methods

Guar gum nanoparticle (GN)

Guar gum nanoparticle was obtained from the laboratory of Dr. Arup Mukherjee, Department of Chemical Technology, University of Calcutta, Kolkata, India where it was prepared by using the protocols previously published (Ghosh *et al.* 2015). The GN was tagged with RITC (absorption maxima: 560 nm; emission maxima: 580 nm) for the *in vitro* and *ex vivo* uptake studies. Untagged GN was used for the *in vivo* studies.

In vitro experiments

Cell Culture

RAW 264.7 macrophage cells were cultured in our laboratory (Immunology & Regenerative Medicine Research Laboratory, Department of Zoology, University of Calcutta, Kolkata, India). The cells were grown in DMEM (Himedia, India), supplemented with 10% FBS (Himedia, India) and 1% Pen-Strep (Himedia, India), and incubated at 37°C with 5% CO_2 in a CO_2 incubator (Thermo Fisher Scientific BB15). When the cells reached 80% confluency, they were passaged into 6-well plates and 96-well plates for further assays.

Cellular uptake and cell viability for GN (RITC tagged guar nano)

a) In vitro treatment of RAW 264.7 cells with GN (for 30 min and 60 min)

1×10^4 RAW 264.7 cells were seeded on gelatin coated coverslips, and allowed to adhere and grow till 70% confluent. The adhered cells were treated with 40 µg/ml RITC-tagged GN for 30 min and 60 min, at 37°C with 5% CO_2. The cells were washed with 1X PBS (Himedia, India), fixed with 4% paraformaldehyde (TCI, Japan) for 20 minutes, washed and counterstained with 300 nM DAPI (Invitrogen, USA). The coverslips were mounted on clean slides and visualized under Andor spinning disk confocal microscope, under the specific set of filters.

b) In vitro inhibitory studies for the mannose receptor mediated uptake in RAW 264.7 cells

Since GN is a galactose-mannose rich compound, to assess whether they are taken up by the macrophages via their mannose receptors or their galactose receptors, a receptor-inhibition study was carried out. $1x10^4$ RAW 264.7 cells were seeded on gelatin coated coverslips, and allowed to adhere and grow till 70% confluent. The adhered cells were treated with 5mM Galactose (Himedia, India), 1mM Mannose (Himedia, India) and 5mM Mannose (Himedia, India) in separate sets

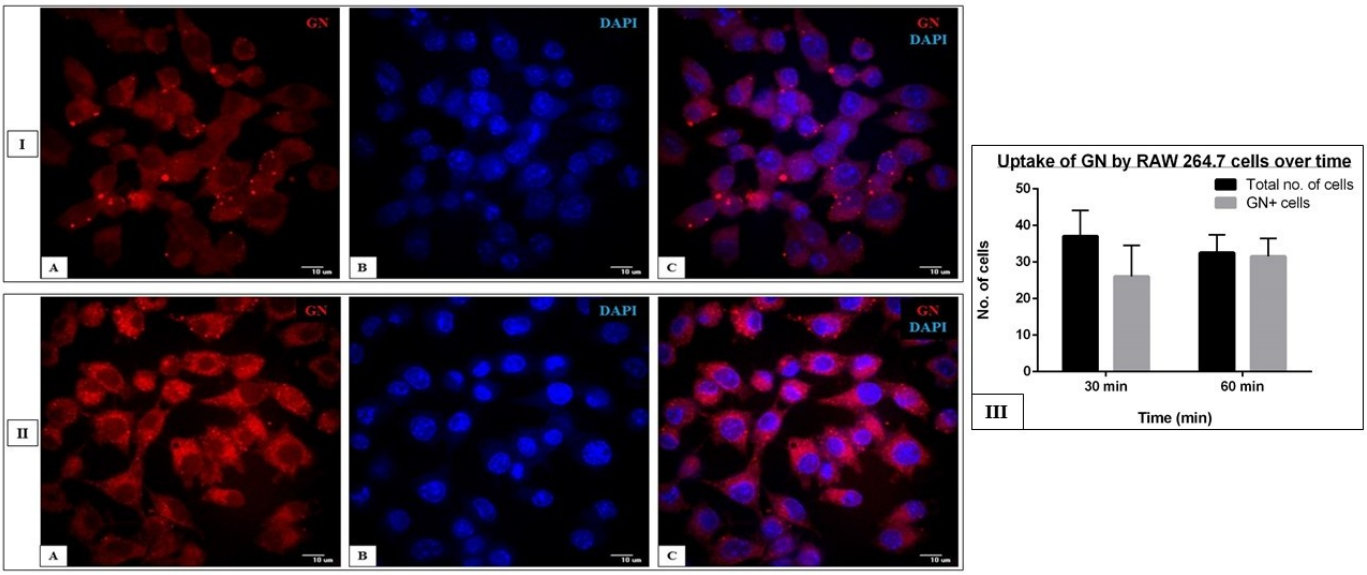

Figure 1. Time dependent uptake of GN by RAW 264.7 cells. RAW 264.7 cells were incubated with GN for (I) 30 mins and (II) 60 mins, counterstained with DAPI and observed under the Andor spinning disk confocal microscope. About 70% of all the cells took up the GN in 30 minutes, while over 96% of all the cells took up the GN in 60 mins. A: Cells stained with GN; B: Cells counterstained with DAPI; C: Merged image. (III) Graphical representation of the number of cells that have taken up GN with time (in 30 and 60 mins) as compared to the total number of cells.

and incubated for 30 mins. Then, they were treated with 40 µg/ml RITC-tagged GN and incubated at 37°C with 5% CO_2 for 1 hr. The cells were washed with 1X PBS (Himedia, India), fixed with 4% paraformaldehyde (TCI, Japan) for 20 minutes, washed and counterstained with 300 nM DAPI (Invitrogen, USA). The coverslips were mounted on clean slides and visualized under Andor spinning disk confocal microscope, under the specific set of filters.

In vitro anti- inflammatory effect of GN Nitric oxide (NO) Assay

1×10^4 RAW 264.7 cells were seeded per well into the wells of a 96-well plate (Nest Biotech, China) in triplicate and incubated for 24 hrs at 37°C with 5% CO_2. One group of cells was treated with 1 µg/ml LPS (Sigma Aldrich) for 2 hrs and the other group without LPS. The cells were then treated with 40 µg/ml GN in respective wells for 1 hr at 37°C with 5% CO_2. 50 µl of 1% Sulfanilamide (SRL, India) in 5% o-phosphoric acid (Merck, India) was added to the wells and incubated at room temperature for 5 minutes in the dark. 50 µl of 0.1% NED [N-1-napthylethylenediamine dihydrochloride (SRL, India)] solution was added to the wells, and incubated at room temperature for 5 minutes in the dark. Absorbance was measured at 540 nm and concentration of NO in the samples was determined from a standard curve.

MTS Assay

1×10^4 RAW 264.7 cells were seeded per well into the wells of a 96-well plate (Nest Biotech, China) in triplicate and incubated for 24 hrs at 37°C with 5% CO_2. One group of cells was treated with 1 µg/ml LPS (Sigma Aldrich) for 2 hrs and the other group without LPS. The cells were then treated with 40µg/ml GN in respective wells and incubated at 37°C with 5% CO_2 for 1 hr. Cell proliferation was assessed using the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit, where 1000µl of MTS reagent was mixed with 50 µl of PMS reagent. 20 µl of this mixture was added to each well, and incubated at

Table 2. Uptake of GN in presence of 5 mM Galactose, 1 mM Mannose and 5 mM Mannose. Galactose was not able to inhibit the uptake of GN as much as mannose. 5 mM mannose was a better inhibitor than 1 mM mannose.

Treatments	High intensity of GN		Low intensity of GN	
	No. of cells	Percentage of total cells	No. of cells	Percentage of total cells
5mM Galactose	9.5 ± 0.5	79.17%	2.5 ± 0.5	20.83%
1mM Mannose	3.5 ± 0.5	38.89%	5.5 ± 1.5	61.11%
5mM Mannose	3.5 ± 2.5	26.92%	9.5 ± 7.5	73.08%

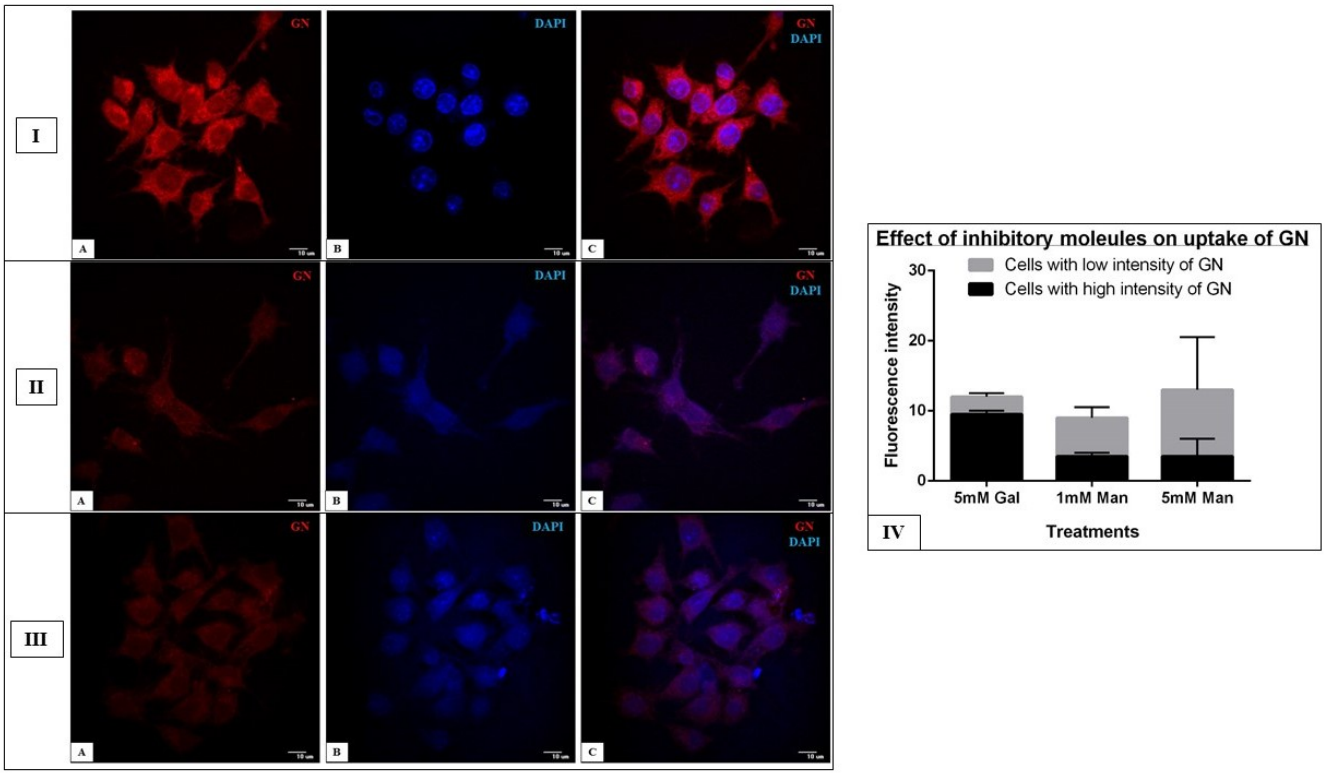

Figure 2. Effect of inhibitory molecules on the uptake of GN by RAW 264.7 cells. RAW 264.7 cells were pre-incubated with (I) 5 mM Galactose, (II) 1 mM Mannose and (III) 5 mM Mannose for 30 mins, followed by GN for 60 mins, counterstained with DAPI and observed under the Andor spinning disk confocal microscope. In (I), many cells showed a high intensity of GN, while few showed low intensity, indicating that there was very little inhibition of GN-uptake by galactose. In (II), few cells showed a high intensity of GN, while many showed low intensity, indicating that there was some inhibition of GN-uptake by mannose. In (III), few cells showed a high intensity of GN, while most of the cells showed low intensity, indicating that 5 mM mannose was better at inhibiting the GN-uptake as compared to both 5 mM galactose and 1 mM mannose. A: Cells stained with GN; B: Cells counterstained with DAPI; C: Merged image. (IV) Graphical representation of the effect of pre-incubation with inhibitory molecules (galactose and mannose) on the uptake of GN by RAW 264.7 cells.

37°C with 5% CO_2 for 3 hours. Absorbance was measured at 490 nm. Considering the proliferation of the untreated cells to be 100%, the corresponding proliferation of the other groups was calculated.

Ex vivo experiments

Uptake of GN by primary culture of peripheral blood cells

After testing the uptake of GN in RAW macrophage cell lines, we wanted to test whether it is also taken up by other cell types. For this, we treated adherent blood cells with GN and immunostained with fluorescent-tagged CD45 (marker for hematopoietic lymphocytes). 10^4 peripheral blood cells from a BALB/c mouse were seeded on 0.2% gelatin-coated coverslips and incubated at 37°C with 5% CO_2. The adherent cells were checked routinely. After the cells reached 70-80% confluence, they were treated with 40 µg/ml RITC-tagged GN in dark and incubated for 2 hrs at 37°C with 5% CO_2 under humidified atmosphere. The adhered cells were washed with 1X PBS (Himedia, India) thrice for 10 minutes and fixed with 4% paraformaldehyde (TCI, Japan) for 20 minutes at room temperature. The cells were washed thrice with 1X PBS for 10 minutes and blocked with 2% BSA (Biosera) in PBS (blocking buffer) for 1 hr at room temperature. 1:100 CD45-PerCP/Cy5.5, diluted in blocking buffer, was added to the cells overnight at 4°C. The cells were rinsed thrice with blocking buffer for 10 minutes away from light, and then counterstained with 300 nm DAPI (Invitrogen, USA) for 15 minutes. The coverslips were mounted on clean slides and visualized under Andor spinning disk confocal microscope, under the specific set of filters.

In vivo experiments

Animals

BALB/c mice (7–9 weeks old, 20–22 g) were purchased from National Institute of Nutrition (NIN), Hyderabad, India. They were housed under specific

Table 3. NO and MTS assay of RAW 264.7 cells in presence of LPS and GN. The NO concentration increased by 1.46 fold ($p<0.05$) with GN alone, by 1.78 fold ($p<0.05$) with LPS alone, and decreased by 1.07 fold with LPS and GN. Cell proliferation increased by 1.22 fold with GN alone, decreased by 1.12 fold ($p<0.05$) with LPS alone, and increased by 1.28 fold ($p<0.05$) with LPS and GN.

Treatments	NO Assay			MTS Assay			
	Concentration of NO (µM)	Fold change, with respect to		Absorbance (540 nm)	Proliferation (%)	Fold change, with respect to	
		RAW - LPS	RAW + LPS			RAW- LPS	RAW+ LPS
RAW	2.90 ± 0.09			0.665 ± 0.097	100		
RAW+LPS	5.17 ± 0.52	(+) 1.78*		0.595 ± 0.027	89.47	(-) 1.12	
RAW+LPS+ GN	4.85 ± 7.30	(+) 1.67	(-) 1.07	0.759 ± 0.022	114.09	(+) 1.14	(+) 1.28*

pathogen free conditions at the animal house of the Department of Zoology, University of Calcutta, and given sterilized water and standard mouse food under 12 hour light and dark cycle throughout the study, according to the rules set by the Institutional & Departmental Ethics Committees. All experiments were performed according to rules laid down by the Institutional and departmental animal ethics committee.

The mice were divided into three groups,

a) Control group: Mice were untreated (n=2).

b) TG group: Mice were treated intraperitoneally with 3% thioglycollate for 24 hours (n=2).

c) TG+GN group: Mice were treated intraperitoneally with 3% thioglycollate, followed by an intraperitoneal treatment of 200 µg GN (n=2).

Treatment of mice

In vivo experiments were done in a mouse model of peritonitis. Peritonitis was induced by intraperitoneal treatment with thioglycollate, which leads to onset of disease within 24 hours, and continues till at least 96 hours. The mice in the TG groups were treated with 200 µl of 3% TG (Himedia, India), dissolved in 1X PBS (Himedia, India) intraperitoneally on Day 0. Mice in the TG+GN group were treated intraperitoneally with 200 µg GN 1 hour after intraperitoneal treatment with 3% TG. The mice were sacrificed 24 hours after treatment by cervical dislocation, and peritoneal fluid and peripheral blood were collected. The peritoneal fluid was collected injecting 1ml 1X PBS, for dislodging the attached cells in the peritoneum. The peripheral blood was collected through cardiac puncture.

Total Cell (TC) Count

The total count and viability of cells in the peripheral blood and peritoneal fluid was checked by Trypan blue staining. Equal volumes of cell suspension and trypan blue (Himedia, India) were mixed and loaded into a haemocytometer, and counted under the microscope. The TC (number of cells/ ml) was plotted.

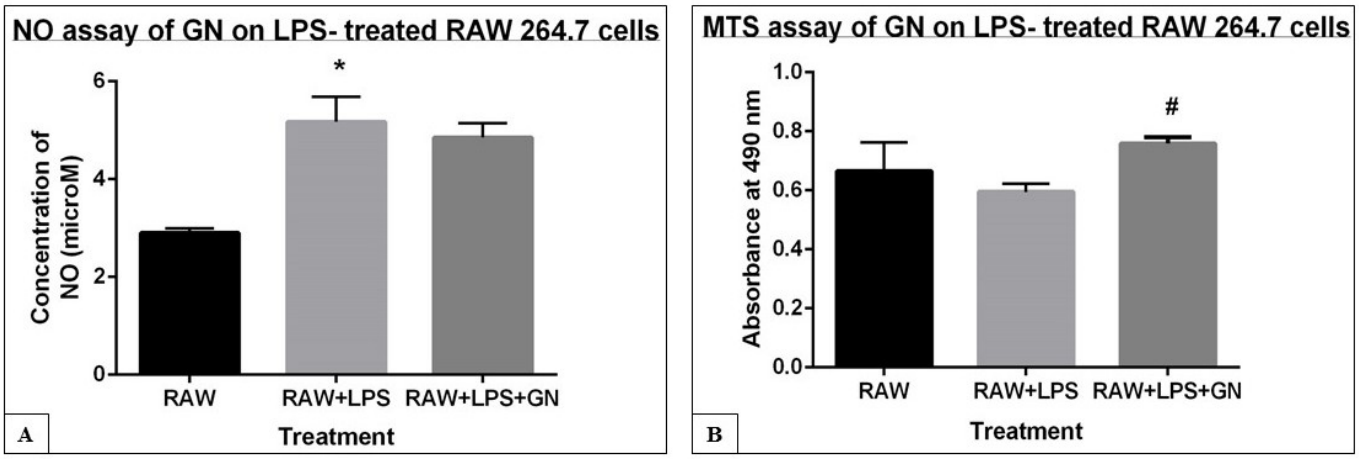

Figure 3. *In vitro* anti-inflammatory effects of GN on LPS- induced RAW 264.7 cells. Effect of treatment with GN on the (A) nitric oxide production by, and (B) on the proliferation of, LPS-induced RAW 264.7 cells. GN showed a 1.07 fold decrease in the NO content, and a 1.28 fold increase ($p<0.05$) in the proliferation. (*: $p<0.05$, with respect to untreated RAW 264.7; #: $p<0.05$, with respect to RAW 264.7 treated with LPS).

Table 4. Total cell count of PB and PF of untreated mice and mice treated with TG and GN. Treatment with TG caused a 1.29 fold increase in TC of PB, while GN caused a 1.28 fold decrease. Treatment with TG caused a 1.87 fold increase ($p<0.05$) in TC of PF, while GN caused a 1.51 fold decrease ($p<0.05$).

Treatments	Peripheral blood (PB)			Peritoneal fluid (PF)		
	TC (x 10^8/ml)	Fold change, with respect to		TC (x 10^6/ml)	Fold change, with respect to	
		Control	TG		Control	TG
Control	2.68 ± 0.38			4.37 ± 0.77		
TG	3.44 ± 1.40	(+) 1.29		8.17 ± 0.07	(+) 1.87*	
TG+GN	2.69 ± 0.99		(-) 1.28	5.40 ± 0.44		(-) 1.51*

Differential Cell (DC) Count

Smears were prepared on microscope slides with peripheral blood and peritoneal fluid. The smears were fixed with methanol, and stained using Giemsa stain (Merck, India) for 15 minutes. The number of monocytes and neutrophils were counted under 40X magnification of a light microscope (Dewinter, India), and the DC (in %) of each cell type was plotted.

CFU assay

For quantification of the clonogenic potential (ability of cells to form colonies), the colony forming unit (CFU) assay was performed in a semi-solid medium using standard protocol. The methyl cellulose semi-solid medium was prepared using IMDM (Himedia, India), FBS (Himedia, India), Pen-Strep (Himedia, India), BSA (Biosera, USA), murine stem cell factor (BioVision, USA) and methyl cellulose (Himedia, India). 500 µl medium was dispensed into the wells of a 24-well plate. Blood and peritoneal fluid cell suspensions were prepared by filtering the cells through a nylon mesh in separate tubes. 500 µl of the prepared cell suspension was then added to the medium in the plate, and incubated at 37°C with 5% CO_2 in a humidified chamber for 7-14 days. The total numbers of colonies were then counted using FLoid Cell Imaging Station (Thermo Fisher Scientific) under 20X magnification, and the clonogenic potential was calculated by dividing the total number of colonies obtained by the number of cells plated.

MTT assay

100 µl of 10^6 cells of peritoneal fluid were plated in the wells (10^5 cells per well) of a 96- well plate and incubated overnight at 37°C with 5% CO_2 in a humidified chamber. The cells were treated with 5µl of 0.5 mg/ml MTT (SRL, India) in 1X PBS, and incubated at 37°C with 5% CO_2 in a humidified chamber for 3 hours. The media was discarded and 150 µl of acidified isopropanol [0.04 M HCl (Merck, India) in isopropanol (SRL, India)] was added to each well and incubated at 37°C with 5% CO_2 in a humidified chamber for 30 minutes. The absorbance was measured at 540 nm. Considering the proliferation of the cells of the untreated control group to be 100%, the corresponding proliferation of the other groups was calculated.

NO assay

50 µl of 10^6 cells of peripheral blood and peritoneal fluid were plated in the wells (0.5 x 10^5 cells per well) of a 96-well plate and incubated overnight at 37°C with 5% CO_2 in a humidified chamber. 50 µl of 1% sulphanilamide (SRL, India) in 5% o-phosphoric acid (Merck, India) was added to the wells for 5 minutes at room temperature in the dark. 50 µl of NED (SRL, India) was added for 5 minutes at room temperature in the dark. Absorbance was measured at 540 nm. The concentration of nitric oxide released by the cells was calculated from a standard curve.

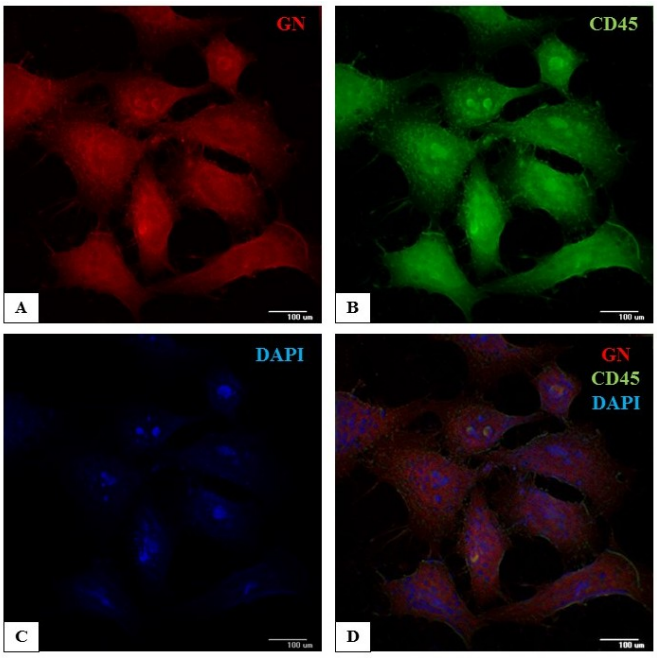

Figure 4. Uptake of GN by primary cells. Peripheral blood cells were incubated with GN for 2 hrs, stained with CD45-PerCP/Cy5.5, counterstained with DAPI and observed under the Andor spinning disk confocal microscope. Almost 100% of the total number of $CD45^+$ and $DAPI^+$ cells took up the GN in 2 hrs. A: Cells stained with GN; B: Cells stained with CD45-PerCP/Cy5.5; C: Cells counterstained with DAPI; D: Merged image.

Table 5. Differential cell count of PB and PF of untreated mice and mice treated with TG and GN. Treatment with TG caused a 1.86 fold increase ($p<0.05$) in neutrophils and a 1.11 fold increase in monocytes of PB; 3.38 fold increase in neutrophils and a 1.50 fold increase in monocytes of PF. GN caused a 1.06 fold decrease in neutrophils and a 1.27 fold decrease in monocytes of PB; 1.40 fold decrease in neutrophils and a 2.85 fold decrease ($p<0.05$) in monocytes of PF.

Treatments	Neutrophils			Monocytes		
	DC (%)	Fold change, with respect to		DC (%)	Fold change, with respect to	
		Control	TG		Control	TG
PB- Control	4.67 ± 0.47			8.49 ± 1.77		
PB- TG	8.70 ± 0.13	(+) 1.86*		9.39 ± 2.04	(+) 1.11	
PB- TG+GN	8.23 ± 0.79		(-) 1.06	7.41 ± 0.03		(-) 1.27
PF- Control	2.17 ± 1.07			8.69 ± 1.00		
PF- TG	7.33 ± 0.88	(+) 3.38		13.02 ± 1.73	(+) 1.50	
PF- TG+GN	5.23 ± 0.10		(-) 1.40	4.57 ± 0.57		(-) 2.85*

Flow Cytometry

Peripheral blood was collected in 1X RBC lysis buffer (Himedia, India), kept at room temperature for 5 minutes, 1X PBS added and centrifuged at 2000 rpm for 5 minutes at 4˚C. The pellet was washed again with 1X PBS (centrifuged at 2000 rpm for 5 minutes at 4˚C). Similarly, peritoneal fluid cells were washed with 1X PBS. The pellets were then stained with two combinations of antibodies: a) 1:50 CD 45- PerCP-Cy5.5 (BioLegend, USA) together with 1:50 CD3- PE (BD Biosciences, India) + 1:50 B220- FITC (BD Biosciences, India) and b) 1:50 CD 45- PerCP-Cy5.5 (BioLegend, USA) together with 1:50 F4/80- PE (Invitrogen, USA) + 1:50 GR1- FITC (MACS Miltenyi Biotec). The pellets were stained for 20 minutes in the dark, centrifuged at 2000 rpm for 5 minutes at 4˚C, and the pellet resuspended in 1X PBS. Flow cytometry was done using FACSVerse (BD Biosciences, USA), where 20000 $CD45^+$ events were gated out from the total events and counted. The flow cytometry results were analysed using the FACSuite software (BD Biosciences, USA).

Statistical Analysis

All data have been presented as Mean ± SEM. Statistical significance has been calculated using t-test, considering all values of $p<0.05$ to be significant. All data have been analysed, and graphs have been prepared, using GraphPad Prism 6 software.

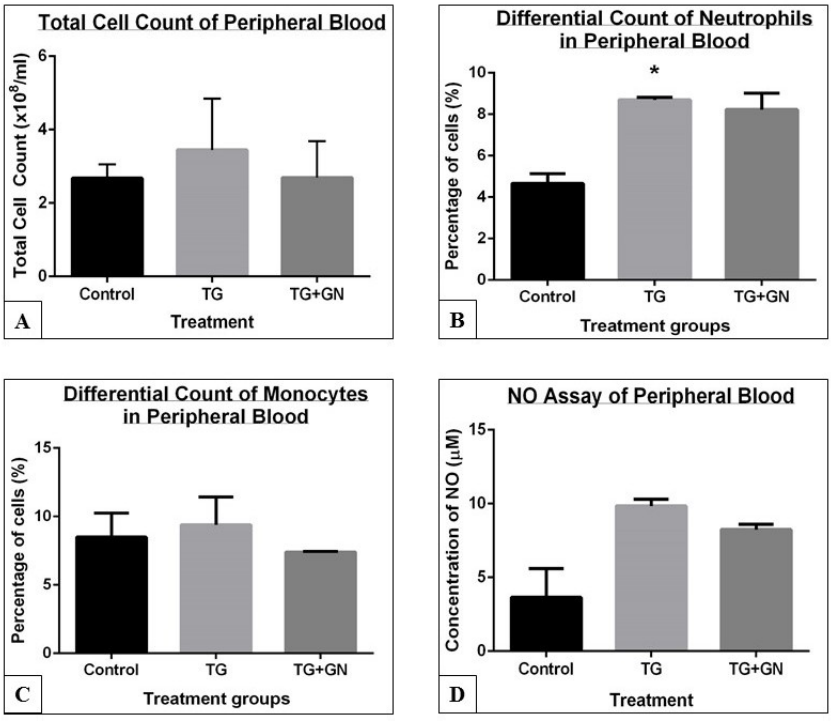

Figure 5. Effect of GN on infiltration and function of peripheral blood cells. Effect of TG and GN on the total cell count of peripheral blood cells (A), as determined using Trypan blue and counted using a haemocytometer, on the differential cell count of neutrophils (B) and monocytes (C) in blood, as determined by Giemsa staining, and observed under 40X magnification, and on the concentration of nitric oxide released by peripheral blood cells (D), as determined by NO assay at 540 nm. (*: $p<0.05$, with respect to untreated control).

Table 6. NO assay of PB and PF of untreated mice and mice treated with TG & GN. Treatment with TG caused a 2.70 fold increase in the concentration of nitric oxide in PB, while GN caused a 1.79 fold decrease. Treatment with TG caused a 1.62 fold increase ($p<0.05$) in the concentration of nitric oxide in PF, while GN caused a 1.54 fold decrease ($p<0.05$).

Treatments	Peripheral blood (PB)			Peritoneal fluid (PF)		
	Concentration (µM)	Fold change, with respect to		Concentration (µM)	Fold change, with respect to	
		Control	TG		Control	TG
Control	3.64 ± 1.95			5.09 ± 0.60		
TG	9.84 ± 0.45	(+) 2.70		8.24 ± 0.15	(+) 1.62*	
TG+GN	8.24 ± 0.35		(-) 1.19	5.34 ± 0.45		(-) 1.54*

Results

In vitro

Time dependent uptake studies for GN with RAW 264.7 cells

RAW 264.7 cells, incubated for 30 minutes with GN, showed a 70% uptake of GN (Figure 1-I). This uptake was increased to 96% when the incubation time was increased to 60 minutes (Figure. 1-II). The uptake was calculated by counting all the DAPI-positive cells which represented the total number of cells and the RITC-positive cells, observed under the Andor spinning disk confocal microscopy. The number of RITC-positive cells among the total DAPI-positive cells gave the percentage of GN uptake (Table 1 and Figure 1-III).

Effect of inhibitory molecules on the uptake of GN

Macrophage cells express mannose receptors, as well as some galactose receptors, on their surface. In order to determine whether the GN was taken up by the macrophages via their mannose or galactose receptors (since GN is a galactose- mannose rich molecule), inhibitory experiments were conducted in which the cells were pre-treated with the free mannose and free galac-

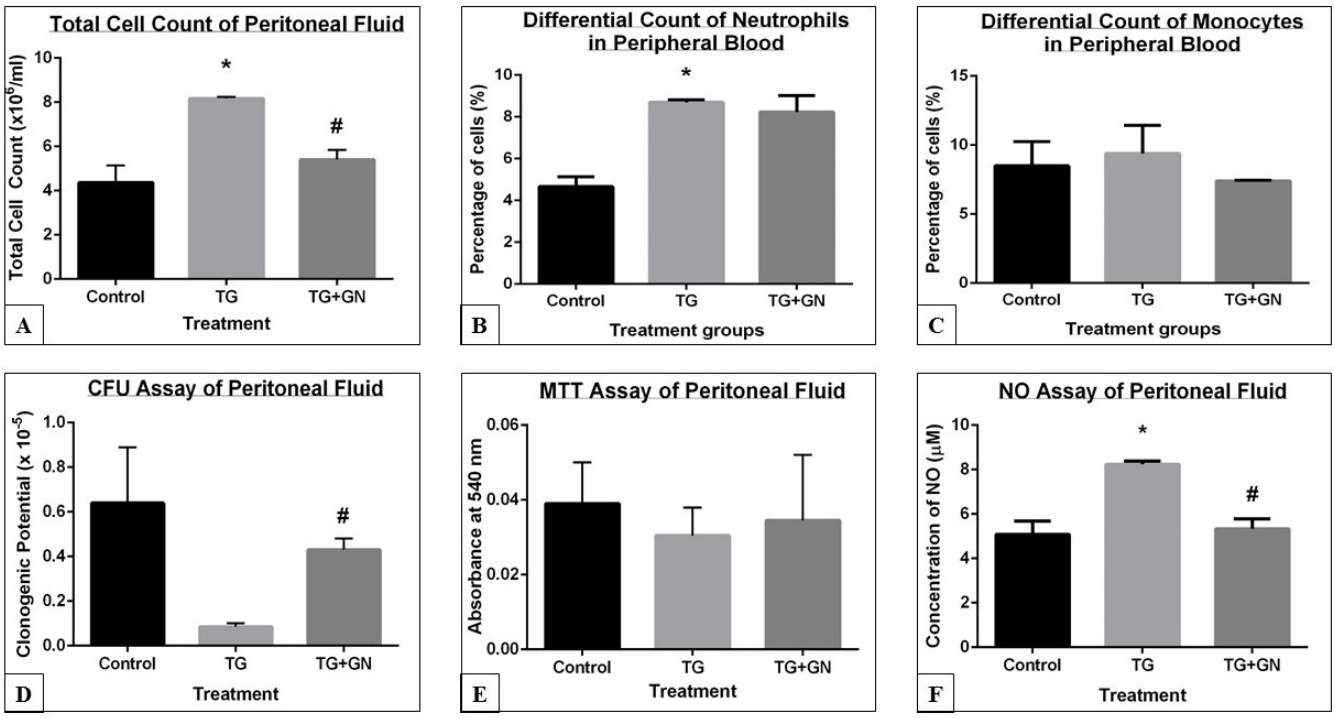

Figure 6. Effect of GN on infiltration and function of peritoneal fluid cells. Effect of TG and GN on the total cell count of peritoneal fluid cells (A), as determined using Trypan blue and counted using a haemocytometer, on the differential cell count of neutrophils (B) and monocytes (C) in PF, as determined by Giemsa staining, and observed under 40X magnification, on the clonogenic potential of PF cells (D) as determined by the CFU assay, on the proliferation of PF cells (E), as determined by the MTT assay at 540 nm, and on the concentration of nitric oxide released by peripheral blood cells (F), as determined by NO assay at 540 nm. (*: $p<0.05$, with respect to untreated control; #: $p<0.05$, with respect to TG- treated).

Table 7. CFU assay of peritoneal fluid of untreated mice and mice treated with TG and GN. Treatment with TG caused a 7.11 fold decrease in the clonogenic potential, while GN caused a 4.78 fold increase ($p<0.05$).

Treatments	Clonogenic Potential ($x\ 10^{-5}$)	Fold change, with respect to	
		Control	TG
Control	0.64 ± 0.25		
TG	0.09 ± 0.02	(-) 7.11	
TG+GN	0.43 ± 0.05		(+) 4.78*

tose before adding GN. The free sugars were rapidly taken up by the cells, thus preventing the galactose-mannose rich GN from entering the cell. Thus the fluorescence of the particles (RITC-positive cells) among the total number of cells (DAPI-positive cells) was measured on the basis of intensity. Cells with low intensity of GN showed some inhibition, whereas the cells with high intensity of GN did not show inhibition. In presence of 5 mM galactose, 79% of cells showed high intensity, while only 20% of the cells showed inhibition (Figure 2-I). Uptake was inhibited by 61% in presence of 1 mM mannose (Figure 2-II) and by 73% in presence of 5 mM mannose (Figure 2-III). Thus 5 mM mannose was effectively blocking the uptake of the mannose rich GN (Table 2 and Figure 2-IV). This may indicate that the GN was indeed taken up by the cells via the mannose receptors, but not via the galactose receptors.

NO Assay and MTS Assay of RAW 264.7 cells

Generally, inflammation leads to an increase in the nitric oxide released by cells. To induce an inflammatory condition, RAW 264.7 cells were treated with LPS. The cells showed a 1.78 fold increase ($p<0.05$) in the NO concentration on treating with LPS as compared to untreated control. In presence of LPS, the cells treated with GN (RAW+LPS+GN) showed a 1.07 fold decrease with respect to the LPS treated cells (RAW+LPS) (Table 3 and Figure 3A). After checking the inflammatory condition, MTS assay was done to estimate the proliferative capacity of cells. In inflammatory conditions, the proliferation of cells reduces, to be restored by therapy. RAW 264.7 cells, on treating with LPS, showed a 1.12 fold decrease in cell proliferation with respect to untreated control. In presence of LPS, the cells treated with GN (RAW+LPS+GN) showed a 1.28 fold increase ($p<0.05$) with respect to the LPS treated cells (RAW+LPS) (Table 3 and Figure 3B).

In vivo

Effect of TG and GN on peripheral blood

At the first stages of inflammation, the body tries to counter the inflammatory attack by its innate immune system. This leads to an influx of cells to the site of inflammation, in this case, the peritoneum. Since the cellular infiltration into the peritoneum occurs via the blood, the number of cells in the peripheral blood also increases. This increase in cells can be assessed by the total cell (TC) count. On treatment with TG, the TC of the peripheral blood (PB) was found to have increased. There was a 1.29 fold increase in the TC of PB of TG-treated group, as compared to the TC of the untreated control. Increase in the TC indicates development of the inflammation. GN treatment successfully reduced the cellular infiltration by 1.28 fold in the PB as compared to the TC of TG- treated mice (Table 4 and Figure 4A). The differential count of neutrophils in the PB of mice treated with TG showed a 1.86 fold increase ($p<0.05$), and monocytes showed a 1.11 fold increase, compared to control. Treatment with GN led to a decrease of 1.06 fold in the neutrophils and a decrease of 1.27 fold in monocytes (Table 5, Figure 4B and 4C), compared to TG- treatment. Nitric oxide released by peripheral blood cells on treatment with TG increased 2.70 fold, and was reduced by 1.19 fold ($p<0.05$) after treatment with GN (Table 6 and Figure 4D). The peripheral blood cells, adhered on gelatin-coated coverslips, also took up GN. Most of the adherent blood cells were $CD45^+$ (hematopoietic lymphocytes). Among these $CD45^+$ cells, almost 100% were also GN^+ (Figure 5). This indicated that GN was taken up non-specifically by all types of lymphocytes, thereby limiting any potential use as an *in vivo* cellular tag.

Effect of TG and GN on peritoneal fluid

The influx of cells into the inflammatory site in the peritoneum was increased due to inflammatory response which occurred after administering TG. There

Table 8. MTT assay of peritoneal fluid of untreated mice and mice treated with TG and GN. Treatment with TG caused a 1.28 fold decrease in the cell proliferation, while GN caused a 1.13 fold increase.

Treatments	Absorbance (540 nm)	Proliferation (%)	Fold change, with respect to	
			Control	TG
Control	0.039 ± 0.011	100.00		
TG	0.031 ± 0.007	78.21	(-) 1.28	
TG+GN	0.035 ± 0.018	88.46		(+) 1.13

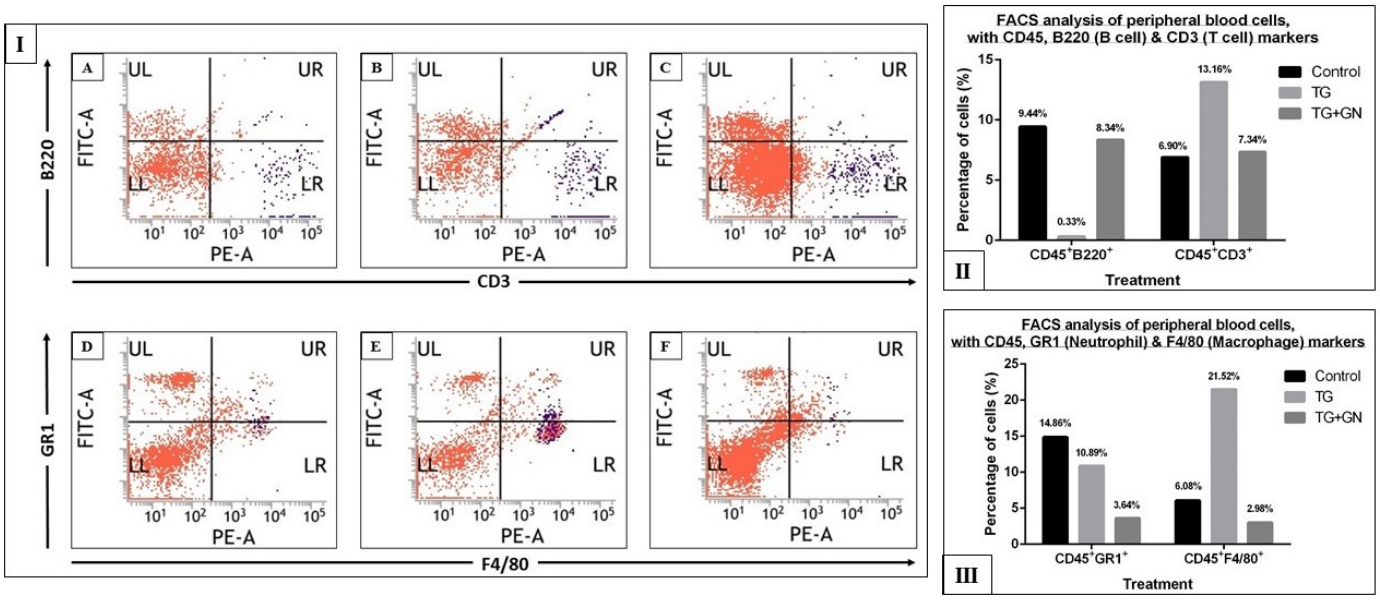

Figure 7. Flow cytometric analysis of peripheral blood done using BD FACSVerse. (I) 20,000 events were counted from gated $CD45^+$ cells (UL: Upper left quadrant; UR: Upper right quadrant; LL: Lower left quadrant; LR: Lower right quadrant). A, D: Control; B, E: TG treated; C, F: TG+GN; A, B, C: B220- FITC vs CD3- PE; D, E, F: GR1-FITC vs F4/80-PE. Graphical representation of the effect of TG and GN on (II) $CD45^+B220^+$ and $CD45^+CD3^+$ cells, and (III) $CD45^+GR1^+$ and $CD45^+F4/80^+$ cells in the peripheral blood.

was a 1.87 fold ($p<0.05$) increase in the TC of peritoneal fluid (PF) of TG- treated group, as compared to the TC of the untreated control. GN treatment successfully reduced the cellular infiltration by 1.51 fold ($p<0.05$) in the PF, compared the TC of TG-treated mice (Table 4 and Figure 6A). In the PF, there was a 3.38 fold increase in neutrophils, and a 1.50 fold increase in monocytes, with TG treatment compared to control. GN treatment was successful in reducing the neutrophils by 1.40 fold and monocytes by 2.85 fold ($p<0.05$), versus the TG-treated mice (Table 5, Figure 6B and 6C). In the peritoneal fluid, the NO concentration increased by 1.62 fold ($p<0.05$) with TG, in comparison to untreated control. NO concentration showed a significant, 1.54 fold ($p<0.05$) decrease with GN treatment, versus TG treatment (Table 6 and Figure 6F).Since the clonogenic potential is the ability of cells to form colonies, it is an indication of the regenerative potential of a therapeutic molecule. In inflammation, the clonogenic potential is found to reduce as compared to untreated cells. A good therapeutic molecule will be able to restore the clonogenic potential of the diseased cells. Treatment with TG showed a 7.11 fold decrease in the clonogenic potential of PF cells as compared to untreated control. GN successfully restored the clonogenic potential compared to TG-treatment, with a 4.78 fold ($p<0.05$) increase (Table 7 and Figure 6D). MTT assay indicates the proliferative capacity of the cells. Proliferation of the PF cells of untreated control, TG-treated and TG+GN-treated cells

Table 9. Flow cytometry of CD45+ cells of peripheral blood of untreated mice and mice treated with TG and GN. PB and PF cells were stained with CD45- PerCP/Cy5.5, B220-FITC, CD3- PE, GR1- FITC and F4/80- PE. B220+ & GR1+ populations in PB decreased with TG and increased with GN (GR1+ decreased with GN), while CD3+ & F4/80+ populations increased with TG and decreased with GN. The B220+ & GR1+ populations in PF decreased with TG and increased with GN, while CD3+ and F4/80+ populations increased with TG and decreased with GN.

Treatments	B Cells (%)	T Cells (%)	Neutrophils (%)	Macrophages (%)
	$CD45^+B220^+$	$CD45^+CD3^+$	$CD45^+GR1^+$	$CD45^+F4/80^+$
PB- Control	9.44	6.9	14.86	6.08
PB- TG	0.33	13.16	10.89	21.52
PB- TG+GN	8.34	7.34	3.64	2.98
PF- Control	1.29	31.3	0.47	40.48
PF- TG	0.98	43.26	0.45	60.45
PF- TG+GN	4.22	22.97	1.25	2.63

were 100%, 78.21% and 88.46%, respectively. With TG treatment, there was a 1.28 fold decrease in the proliferation as compared to control. Proliferation was restored to some extent (1.13 fold, with respect to TG-treated) by treatment with GN (Table 8 and Figure 6E).

Changes in B220, CD3, GR1and F4/80 population in peripheral blood and peritoneal fluid

To assess the effect of GN on the infiltration of B cells, T cells, neutrophils and macrophages, flow cytometry was done. CD3 has been used as a marker for T cells, while B220 has been used as a marker for B cells. Neutrophils are typically $CD45^+GR1^+F4/80^-$, while macrophages are $CD45^+GR1^-F4/80^+$. Inflammatory monocytes are $GR1^+F4/80^+$.

Peripheral Blood: As we see in Figure. 7, and Table 9, there was a larger population of $B220^+$ cells (Figure 7-I-A) and $GR1^+$ cells (Figure 7-I-D) in the control as compared to TG (Figure 7-I-B and Figure 7-I-E) and TG+GN (Figure 7-I-C and Figure 7-I-F). However, while the population of $B220^+$ has increased again in TG+GN, as compared to TG, the population of $GR1^+$ has further reduced. The population of $CD3^+$ cells (Figure 7-I-A) and $F4/80^+$ cells (Figure 7-I-D) was much low in control than in TG- treated (Figure 7-I-B and Figure 7-I-E), which has again reduced in TG+GN (Figure 7-I-C, Figure 7-I-F, Figure 7-II and Figure 7-III).

Peritoneal Fluid: According to Figure. 8, and Table 9, there is a small population of $B220^+$ cells (Figure 8-I-A) and $GR1^+$ cells (Figure 8-I-D) in the control, and a smaller population in TG (Figure 8-I-B and Figure 8-I-E). There is a comparatively larger population in TG+GN (Figure 8-I-C and Figure 8-I-F). The population of $CD3^+$ cells (Figure 8-I-A) and $F4/80^+$ cells (Figure 8-I-D) is lower in control than in TG-treated (Figure 8-I-B and Figure 8-I-E), which has again reduced in TG+GN (Figure 8-I-C, Figure 8-I-F, Figure 8 -II and Figure 8-III).

In both cases, as expected, there was an increase in the population of $F4/80^+$ macrophages with TG treatment. GN has reduced this population, both in the PB and in PF. There was also a large decrease in the population of the $B220^+$ cells with TG, which has again been somewhat restored with GN. The $CD3^+$ cell population has increased with TG and again decreased with GN in both PB and PF. However, while the population of $GR1^+$ cells has decreased with TG in both PB and PF, it has further decreased with GN in the PB, but has increased in the PF.

Discussion

Nanoparticles show promising features for pharmaceutical applications, especially for targeted drug delivery and bioimaging. The biomedical applications require a detailed understanding of interactions between nanoparticles and biological systems (Brandenberger

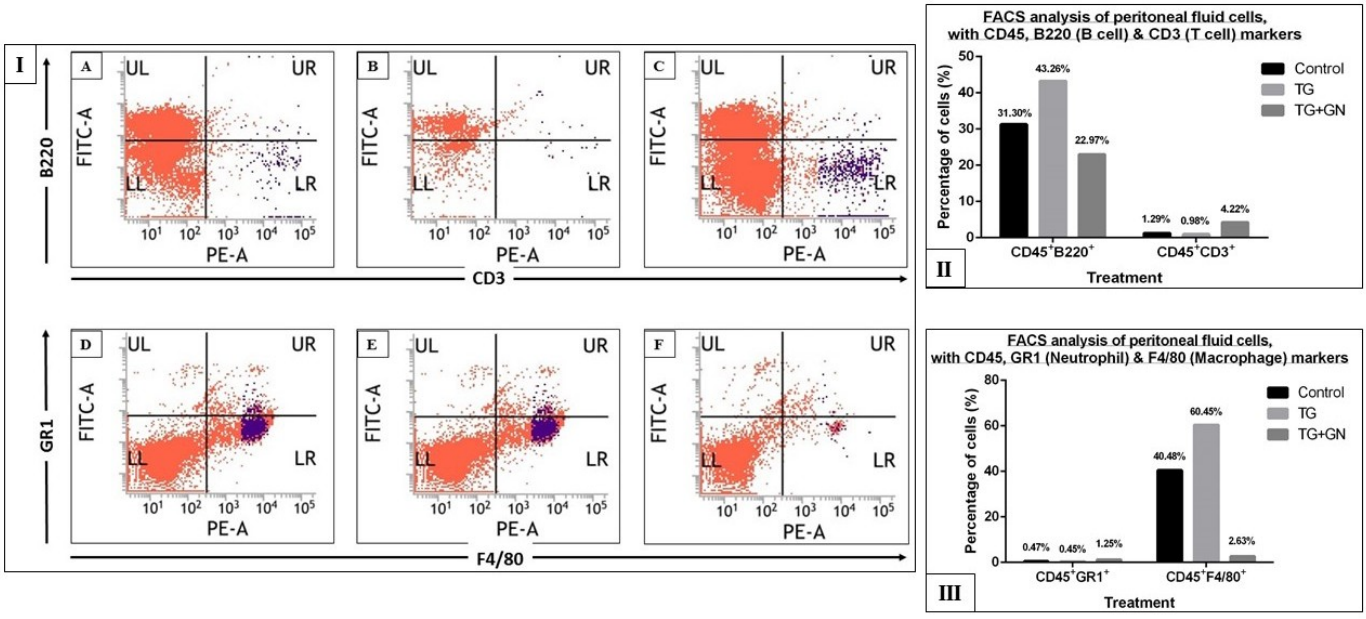

Figure 8. Flow cytometric analysis of peritoneal fluid done using BD FACSVerse. (I) 20,000 events were counted from gated $CD45^+$ cells (UL: Upper left quadrant; UR: Upper right quadrant; LL: Lower left quadrant; LR: Lower right quadrant). A, D: Control; B, E: TG treated; C, F: TG+GN; A, B, C: B220- FITC vs CD3- PE; D, E, F: GR1-FITC vs F4/80-PE. Graphical representation of the effect of TG and GN on (II) $CD45^+B220^+$ and $CD45^+CD3^+$ cells, and (III) $CD45^+GR1^+$ and $CD45^+F4/80^+$ cells in the peritoneal fluid.

et al. 2010). Guar gum is a non-toxic and biodegradable polymer with various applications such as emulsifier, suspending and bioadhesive agent (Ahuja *et al.* 2012). The guar gum nanoparticle (GN) used in this study was designed for a biomedical application. Upon exposure, nanoparticles interact with cells and enter into the cells by various mechanisms depending on their size, material, and surface characteristics.

In this study, we have performed both *in vitro* and *in vivo* experiments with guar gum nanoparticle to understand its therapeutic effect against inflammatory diseases. Cellular uptake was seen in adherent macrophage cells, where the unbound particles were washed off before microscopic observation to reduce their interference in the microscopic studies. We performed MTS cell proliferation assay and nitric oxide assay to determine whether GN has any anti-inflammatory effect. Our experiments showed potential anti-inflammatory and pro-regenerative activities of GN. As macrophages have special affinity for the carbohydrate-rich domains, due to the presence of C-type lectin receptors (mannose and galactose receptors) expressed on them (Kawasaki *et al.* 1986, McKenzie *et al.* 2007, Suzuki *et al.* 1996), we also performed receptor inhibition studies using mannose and galactose to see whether the GN was taken up by the cells via these receptors. Free mannose and galactose were used as competitive inhibitors for the uptake of GN, with the idea that, the different concentrations of free mannose and galactose would inhibit the uptake of the galactomannan nanoparticle by the macrophage cells through their receptors. The competitive binding of the free mannose and galactose to the nanoparticles with respect to the mannose/ galactose-rich nanoparticles would reflect the specificity of the uptake of nanoparticles in the cells. Our experiments, however, showed that the presence of exogenous mannose in the medium led to greater inhibition of GN-uptake than the presence of galactose. Thus, it can be said that, the uptake of GN was indeed occurring through the mannose receptors on the cells. The structural characterization of the mannosylated GN plays a significant role in the macrophage specific delivery of the nanoparticle. Different studies demonstrated that, engineered nanoparticles could penetrate cell membranes and transport into cells of various types (Zhao *et al.* 2011).

One of the main concerns in the development of nanomedicines for disease diagnoses and therapy is to ensure that the designed nanoparticles can easily enter the cells. This is critical for the nanoparticles to be able to execute their diagnostic or therapeutic functions *in vivo*. The processes leading to nanoparticles penetrating the cell membrane directly relate to their cellular uptake. Experimental results indicate that engineered nanoparticles could penetrate cell membranes and transport into cells of various types, such as endothelial cells, pulmonary epithelia, intestinal epithelia, alveolar macrophages, and neuron cells (Akerman *et al.* 2002, Florence *et al.* 2001, Hoet *et al.* 2001, Kato *et al.* 2003, Oberdorster *et al.* 2002).

As the GN uptake was observed in the macrophage cell line (RAW 264.7 cell) *in vitro*, we were interested to study its uptake by specific cell types among different cells. This was achieved by culturing primary cells obtained from murine peripheral blood, obtained from BALB/c mice. We used the CD45 marker for screening hematopoietic cells among the mixed population of blood cells. Immunostaining GN-treated cells with CD45-PerCP/Cy5.5 and counter-staining with DAPI led to the observation that almost 100% of the $CD45^+$ cells had taken up the GN. This reflected that, GN uptake was not specific for a particular type of cells and it was non-specifically taken up by all hematopoietic lymphocytes.

Finally, we have performed an *in vivo* experiment, in a murine model of thioglycollate- induced aseptic peritonitis. We treated mice (previously treated intraperitoneally with 3% thioglycollate to induce peritonitis) intraperitoneally with 200 μg/ml GN. After 24 hours, the mice were sacrificed, and peripheral blood (PB) and peritoneal fluid (PF) were collected. At the first stages of inflammation, the body tries to counter the inflammatory attack by its innate immune system. This increase in cells was assessed by the total cell (TC) count which indicates the development of inflammatory pathway. GN treatment successfully reduced the cellular infiltration in the PB and PF, compared the TC of TG- treated mice (Table 4, Figure 4A and 6A). CFU assay demonstrated that, GN successfully restored the clonogenic potential of TG-induced diseased cells (Table 7 and Figure 6D), showing that the GN has helped the cells regain their potential to proliferate, which was also corroborated by the MTT assay (Table 8 and Figure 6E). One of the main results of an inflammatory reaction in a body is the overproduction of pro-oxidative agents, like nitric oxide (MacMicking *et al.* 1997, Rahman 2007). In this work, *in vivo* nitric oxide assay also showed a significant decrease of NO concentration with GN treatment as compared to the NO concentration of the TG-induced diseased peritoneal fluid cells (Table 6 and Figure 6F). Flow cytometric analysis showed a marked reduction in the population of macrophages in both the PB (Table 9 and Figure 7) and the PF (Table 9 and Figure 8), with GN treatment. The results confirmed the effectiveness of guar gum nanoparticles against inflammatory disease.

Conclusion

The increasing interest of the technology of drug form in natural biopolymers has become the reason for undertaking investigations on the possibility of guar gum application. The *in vitro* studies performed with the RAW 264.7 cells showed successful uptake of the mannose rich guar gum nanoparticle in a short duration of time. *In vivo* experiment in a murine model of thioglycollate-induced aseptic peritonitis showed that, GN decreased the symptoms of the disease, including the total cell count, the differential count of neutrophils and monocytes, and the nitric oxide released by the cells. On the other hand, it has managed to increase the clonogenic potential of the cells as well as their proliferative capacity. In the context of our current study, we can conclude that, GN can be used as an anti-inflammatory agent against inflammatory diseases. Continued research in this topic will provide a better understanding of the therapeutic effect of guar gum nanoparticle and will undoubtedly help the design of safe and effective nanoparticle platforms for biomedical applications.

Acknowledgements

We wish to acknowledge Professor Arup Mukherjee, Department of Chemical Technology, University of Calcutta and his Research Fellow Mr. Sumanta Kumar Ghosh for preparing Guar gum nanoparticles and providing us with it. We wish to acknowledge the Indian Council for Medical Research (ICMR), New Delhi, for providing a fellowship to NG & SB, and the University Grants Commission (UCG), New Delhi, for providing fellowship and contingency grant to SM. We also wish to acknowledge UGC-SAP, UPE-Phase II and DST-FIST for providing departmental infrastructure. We acknowledge the Indian Institute of Chemical Biology (IICB), Kolkata, for allowing us the use of the Andor Spinning Disk Confocal Microscope. We also acknowledge the BD- CoE at the Centre for Research in Nanoscience & Nanotechnology (CRNN), University of Calcutta, Kolkata, for allowing us to use the BD FACSVerse. This work was funded from the corresponding author's grant with the Indian Council of Medical Research (ICMR), New Delhi.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

NG and SM performed the experiments, analysed the data and wrote the manuscript. SB gave valuable input to the manuscript. ERB initiated the project with her idea, designed the experiments, analyzed all data and wrote the manuscript.

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