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Neem Leaf Glycoprotein Facilitates Lung Carcinoma-Associated Antigen-Specific Anti-Cancer Immune Response Utilizing Macrophage-Mediated Antigen Presentation and Induction of Type 1 Cytokines Coupled with Nitric Oxide Production

Nim Ağacı Yaprağı Glikoproteini Akciğer Karsinomu ile İlişkili Antijenlerin Makrofajlar Tarafından Yapılan Antijen Sunumu ile Oluşturulan Yanıtını Kolaylaştırır ve Tip 1 Sitokinler ile Nitrik Oksidin Salınımını Artırır

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Abstract

Introduction: Based on earlier observations on unique immunomodulatory and adjuvant functions of neem leaf glycoprotein (NLGP), investigations of this work were designed. NLGP was attempted to be used as an adjuvant for lung carcinoma-associated antigen (LCA) which not only activated macrophages but also induced macrophages to release nitric oxide (NO), a key tumoricidal agent known to regulate T-cell proliferation, cytokine production, cell signaling, and apoptosis.

Materials and Methods: Macrophages, generated from peripheral blood mononuclear cells (PBMCs), were pulsed with LCA isolated from lung carcinoma cell line A549, in presence or absence of NLGP for antigen presentation. Intramacrophageal NO was estimated based on Griess reaction. Cytokine levels were estimated by ELISA. Lymphocytic proliferation was checked by MTT assay. Cytotoxic T lymphocytes (CTLs) generated cytotoxicity was tested by LDH assay.

Results: NLGP potentiates immune responses during pulsation with LCA by specific lymphocytic proliferation ($p<0.001$) and generation of CTLs ($p<0.001$). LCA+NLGP treatment creates a type-1 immune environment by increasing secretion of type-1 cytokines IFN-γ and IL-12 ($p<0.001$) and decrease in type-2 cytokines IL-4 and IL-10 ($p<0.001$). LCA+NLGP treatment increased the release of type-1 cytokine-dependent NO. *In vitro* neutralization of IFN-γ/IL-12 results into drastic decrease in NO release from macrophages.

Conclusion: Obtained results demonstrated the interdependence of three anti-tumor immune functions, namely, NO production, CTL generation and production of a type-1 immune response mediated through NLGP. NLGP-generated anti-LCA immune response would be an effective strategy to treat lung carcinomas.

Keywords: Neem Leaf Glycoprotein (NLGP), Nitric Oxide (NO), Cytotoxic T lymphocytes (CTLs)

Öz

Giriş: Nim ağacı yaprağı glikoproteininin (NAYG) benzersiz düzenleyici ve adjuvan fonksiyonlarına ilişkin daha önceki gözlemlerine dayanarak, araştırmacılar bu çalışmayı tasarlamıştır. NAYG'nin sadece makrofaj aktivasyonunda değil, aynı zamanda T hücre proliferasyonu, sitokin üretimi, hücre sinyal iletimi ve apoptozisi düzenlediği bilinen önemli bir tümör öldürücü ajan olan nitrik oksiti (NO) salgılayan makrofajları indükleyen akciğer karsinomu ile ilişkili antijen (LCA) için bir adjuvan olarak kullanılması planlanmıştır.

Gereç ve Yöntemler: Periferik kan mononükleer hücrelerden (PKMH'ler) elde edilen makrofajlar, antijen sunumu için NAYG varlığında veya yokluğunda, akciğer karsinomu hücre dizisi A549'dan izole edilmiş LCA ile uyarıldı. Makrofaj içi NO Griess reaksiyonu kullanılarak saptandı. Sitokin seviyeleri ELISA ile ölçüldü. Lenfosit proliferasyonu MTT testi ile saptandı. Sitotoksik T lenfositleri (CTL'ler) sitotoksik aktivitesi LDH metodu ile saptandı.

Sonuçlar: NAYG, LCA ile uyarılmış lenfositlerin bölünme hızını ($p<0.001$) ve sitotoksik T lenfosit yanıtını ($p<0.001$) arttırdı. LCA ile birlikte NAYG'nin uygulanması tip 1 sitokinlerden IFN-γ ve IL-12 salgılanmasında artışa ($p<0.001$), tip 2 sitokinlerden IL-4 ve IL-10 üretiminde düşüşe ($p<0.001$) neden oldu. LCA+NAYG uygulaması ise tip-1 sitokinlere bağlı NO üretimini artırmasına karşılık, *in vitro* IFN-γ/IL-12 nötralizasyonu makrofajlardan NO salınımını belirgin derecede azalttı.

Sonuç: Elde edilen sonuçlar, NO üretimi, sitotoksik T lenfosit oluşumu ve tip-1 bağışık yanıt gelişimi olmak üzere üç anti-tümör immün fonksiyonunun NAYG ile birbirine bağımlılığını göstermiştir. NAYG ile oluşturulmuş anti-LCA immün yanıtı, akciğer karsinomlarının tedavisinde etkin bir strateji olarak kullanılabilir.

Anahtar Kelimeler: Nim ağacı yaprağı glikoproteini (NAYG), nitrik oksit (NO), sitotoksik T lenfositleri (CTLs)

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Introduction

Lung cancer continues to be a major cause of death to date, causing 1.2 million deaths/year worldwide.[1,2] Recent studies have attempted in identifying, lung carcinoma-associated antigen. The tumor escape mechanisms have also been studied and a number of vaccines have been approved for market for treating lung cancer.[3] Despite advances in the development of cancer vaccines, results from clinical trials have not been satisfactory.[2,3]

Immunotherapy has been shown as an attractive option since the generation of specific anti-tumor immune responses by identifying the tumor-specific antigens favors tumor cell death with minimal impact on normal tissue.[3]

Neem leaf glycoprotein (NLGP) is a nontoxic adjuvant[4] which has shown anti-cancer immune response by effective antigen presentation in three tumor antigens: B16 melanoma antigen[5], breast tumor-associated antigen[6] and carcino-embryonic antigen (CEA).[7]

Differentially pulsed macrophages consist of a heterogeneous population of cells with functional plasticity[8] that have an essential role in the events of inflammation and also tissue homeostasis.[9]

In the event of pathogen entry, or growth of tumor cells in the body, the macrophages receive a stimulus from the microenvironment. Upon receiving the stimuli, the activated macrophages differentiate into different sub-population of cells, which are capable of destroying pathogens, tumor formation and promote healing and tissue repair in the resolution phase of inflammation.[10–12]

Classically activated macrophages are proficient effectors in promoting Th1 type immune response and in fighting against bacterial infections as well as malignant tumors.[12] On the other hand, macrophages stimulated by Th2 related cytokines such as IL-4 or IL-13 and IL-10, immune complexes, and glucocorticoids, are grouped as alternative activated (M2) macrophages.[13]

Th1 and Th2 cells obtained from same precursor T cells differentiate into two distinct lineages, principally in a microenvironment, influenced due to Th cell-specific cytokines.[12] Cytokines IL-12 and IL-4 are imperative in facilitating the differentiation of Th1 and Th2 cells respectively.[14,15]

Th1 cells help to clear out intracellular pathogens and are closely associated with inflammatory diseases.[15] Th2 cells, on the other hand, help in eradicating the extracellular parasites and reduce allergy in the body.[16]

This dichotomy of function between the Th1 and Th2 cells is essential in maintaining the balance of immune response and thus forms the basis of the current concept in developing immune therapies for targeting various diseases.[16,17]

Given the close relation between Th1 cells and nitric oxide in the elimination of disease, it is very likely that there exists a reciprocal regulatory mechanism between them.[16]

Nitric oxide (NO), is known to be a key tumoricidal agent which regulates T-cell proliferation, cytokine production, cell signaling, and apoptosis.[17] Several other studies have shown that the T-cell derived cytokines IFN-γ, along with IL-2 and/or TNF-α, are macrophage activators and serve as potent inducers of NO.[18]

NO has been shown to follow a mechanism that selectively enhances Th1 and not Th2 cell proliferation.[16] NO showed its effect, not only by acting directly on T cells but also in synergy with IL-12 produced by antigen presenting cells.[19]

In previous studies, NLGP has been shown to assist in the activation of macrophage and in the release of nitric oxide (NO).[7] In addition, NLGP has also been shown to promote NO-dependent type-1 immune response, with increased production of type-1 cytokine IL-12 and inhibiting the release of type-2 cytokines IL-4 and IL-10.[20,21]

However, any study has not been carried out with NLGP and its effect on lung carcinoma-associated antigen. In this study, we attempt to show the Th1 dependent anti-tumor immune response generated by NLGP assisted macrophage-dependent antigen presentation of LCA, following NO release and cytotoxic T lymphocyte (CTL) generation.

Materials and Methods

Antigen, antibodies, and reagents

Primary antibodies mouse anti-human IFN-γ (Cat#14–7311–81) and IL-12 (Cat#14-7125-81) were purchased from eBioscience, USA and mouse anti-human IL-4 (Cat#500703) and rat anti-human IL-10 (Cat#501409) from Biolegend, San Diego, CA, USA. Secondary antibody

goat anti-mouse IgG conjugated to HRP (horseradish peroxidase) (Cat#sc-2055) was purchased from Santa-Cruz, Texas, USA for cytokine profiling TMB substrate was purchased from BD-Pharmingen, USA. Cytotoxicity Detection Kit (LDH) was obtained from Roche Diagnostics, Mannheim, Germany. RPMI 1640 medium for culturing cells and fetal bovine serum (FBS), penicillin, streptomycin were purchased from Life Technologies, NY, USA. DMSO (dimethyl sulphoxide), Bovine serum albumin (BSA), Tween-20, TritonX-100, ConA (Concanavalin-A), MTT (3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) were acquired from Sigma, USA.

Cell Line

A549 lung carcinoma cell line was obtained from National Centre for Cell Science, Pune, India. A549 cells were maintained in Ham's F12 k medium containing 10% new born calf serum containing 100 µl/ml of penicillin and streptomycin 100 µL/mL in a humidified atmosphere of 5% CO_2 incubator at 37°C.

Neem leaf glycoprotein

Extract from neem (*Azadirachtaindica*) leaves was taken from a standard source and was prepared by the method as described earlier.[22] Leaf powder, which was shed-dried and pulverized, was soaked overnight in phosphate buffered saline solution (PBS), pH 7.4. The supernatant was then collected after centrifugation at 1500 rpm. The extract was then extensively dialyzed against PBS, pH 7.4 and concentrated by Centricon Membrane Filter (Millipore Corporation, Bedford, MA, USA) with 10 kDa as cut off. The endotoxin content of the freshly prepared neem leaf extract was determined by Limulus Amebocyte Lysate (LAL) test as per manufacturer's (Salesworth India, Bangalore, India) instruction. The endotoxin content of all batches which were prepared was found to be lesserthan 6 pg/ml. The active component of this preparation is a glycoprotein, as characterized earlier[23] and was thus termed as neem leaf glycoprotein (NLGP). The protein concentration of NLGP solution was measured by Lowry's protein estimation method using Folin's Phenol reagent.[24]

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

The peripheral blood mononuclear cells (PBMC) from healthy donors (with informed consent) were isolated from heparinized venous blood by density gradient centrifugation. 2 mL of blood was diluted with 2 mL of 1X phosphate buffer saline (PBS). 2 mL of Ficoll Paque (obtained from GE Health Care) was taken in a falcon tube and 4 mL of diluted blood was layered on it. It was centrifuged at 2200 rpm for 30 minutes in fixed-angle rotor without brake. A ring-like foggy monolayer of PBMC was formed at the Ficoll-plasma interface. This was withdrawn using micropipette. The monolayer was then transferred to a new Falcon tube. Then 5 mL PBS (pH=7.4) was added for washing the monolayer cells after which the cells were centrifuged at 1500 rpm for 10 minutes and the supernatant was discarded. The streaky pellet, thus obtained was re-suspended in 500 µL of Roswell Park Memorial Institute (RPMI 1640) media and transferred to a 12-well plate. 500 µL of fresh RPMI media, 10% fetal bovine serum (FBS) (50 µL), 10 µg/mL of Penicillin and Streptomycin and 5 ng/mL macrophage colony stimulating factor (MCSF) was added to the 12 well-plate and incubated in 5% CO_2, 37°C for 48 hours.

Neem leaf glycoprotein treatment

The non-adherent cells were removed and adherent cells were scraped. Non-adherent and adherent cells were thoroughly washed and cultured in RPMI 1640 (Life Technologies, New York, NY), supplemented with 10% FBS, penicillin (50 units/ml), streptomycin (50 g/ml) at 37°C in a humidified atmosphere with 5% CO_2, with and without NLGP (2.5 µg/mL).

Activation of macrophages with LCA

Irradiated macrophages isolated from healthy donors were suspended into plastic Petri plates at a concentration of 1×10^6 cells/ml in complete RPMI 1640 media in the presence of either LCA (5 µg/mL) or LCA (5 µg/mL) + NLGP (2.5 µg/mL) for 24 h at 37°C. This culture was further used for various *in vitro* assays. The effect of NLGP on autologous and allogenic lymphocyte proliferation was studied using the MTT assay. Concanavalin A (ConA) which is a non-specific stimulator of lymphocytes was used as the positive control.

Nitric oxide (NO) release assay

Accumulation of nitrate in the culture supernatant was measured based on Griess reaction, which is a colorimetric assay. In brief, aliquots (50 µL) of the culture supernatant were taken and incubated with an equal volume of Greiss reagent (1% sulphanilamide, 0.1% naphthyl ethylene dihydrogen chloride, and 2.5% orthophosphoric acid) at room temperature for 10 minutes, and nitric oxide concentration was determined by absorbance at 550 nm with respect to the sodium nitrite standard.

Proliferation assay

LCA pulsed irradiated macrophages co-cultured with lymphocytic cells (2×10^5 cells/well) were plated in each well of a 96 well microtiter plate and stimulated with LCA (5 µg/well). A nonspecific stimulator-ConcanavalinA (ConA) was used as positive control (1 µg/well) for 72 h. The cell proliferation was checked by MTT assay as described. In brief, 20 µL aliquot of MTT solution (5 mg/mL) was added in the cell culture and incubated for 4 h at 37°C. The medium was removed by aspiration and the purple colored formazan precipitate formed was dissolved in DMSO solution (100 µL) and the absorbance was measured at 550 nm using a microplate reader. The absorbance obtained was directly proportional to the number of viable cells.

Cytotoxic T Lymphocyte (CTL) assay

Cytotoxic T lymphocytes (CTLs) *i.e.* effector cells were generated on LCA treatment were cultured overnight with target A549 cells at an Effector: Target ratio of 10:1 at 37°C in round bottom, 96-well plate. Macrophages were pulsed with or without tumor antigen in presence or absence of NLGP and they were irradiated to co-culture with T cells and A549 cells. The cytotoxicity was assessed by the release of lactate dehydrogenase (LDH) using a commercially available kit. The absorbance was measured at 490 nm using a microplate reader (Tecan Spectra, Grodig, Austria).

Cytokine ELISA

The extracellular release of cytokines (IFN-γ, IL-12, IL-4, and IL-10) from co-culturing differentially pulsed irradiated macrophages was determined by analyzing culture supernatants by ELISA in indirect format as described earlier.[25] 96-well microtiter plates were coated with 100 µL of cell-free supernatant, incubated overnight at 4°C and blocked with 5% bovine serum albumin for 2 h. After washing, a primary antibody in 1:100 dilution ratio (anti-mouse IFN-γ, IL-12, IL-4, and IL-10) was added to each well and incubated overnight. Bound cytokines were detected by staining with peroxidase-conjugated secondary antibody in 1:1000 dilution (anti-mouse IgG). The color was developed using tetramethylbenzidine (TMB) substrate solution. The reaction was stopped with 1N H_2SO_4 and absorbance was measured at 450 nm using a microplate reader (Tecan Spectra, Grodig, Austria). A standard curve was generated for each type of cytokine by plotting the mean absorbance of each standard on the y-axis against the concentration on the x-axis to get a best fit curve through the points on the graph. The data was linearized by plotting the log of the each type of cytokine concentrations versus the log of the optical densities and the best fit line was determined by regression analysis. Detection limits of IFN-γ, IL-12, IL-4 and IL-10 were range between 15.625–1000 pg/mL, 7.8–500 pg/mL, 31.3–2000 pg/mL and 7.8–500 pg/mL respectively.

Neutralization Assay

In the cell culture, anti-mouse IFN-γ, IL-12, IL-10, and IL-4 were added along with stimulants *i.e.* (LCA, LCA+NLGP) and incubated at 37°C in a humidified atmosphere for 48 h. Following incubation, the supernatants were collected to measure NO released.

Statistical analysis

All results represent the average of 3–5 separate *in vitro* experiments involving triplicate assays. The statistical significance was established by using one-way ANOVA using INSTAT3 Software.

Results

NLGP treatment augments antigen-specific proliferation of lymphocytes

NLGP induced better proliferation of lymphocytes (Figure 1) NLGP, even in absence of LCA had a positive impact on the production of lymphocytes as the number of cytotoxic T lymphocytes generated, were capable of clearing tumor more effectively.

NLGP enhances production of nitric oxide from macrophages, thereby generating LCA specific immune response *in vitro*

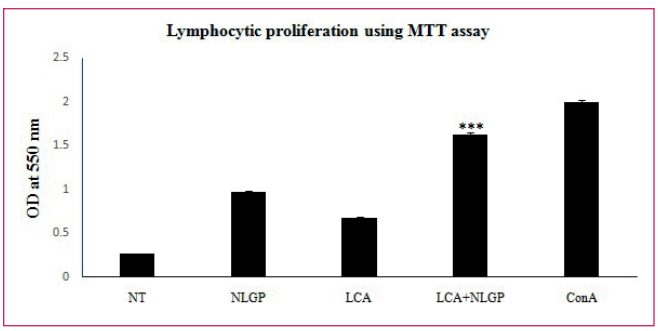

Figure 1. Lymphocytes isolated from peripheral blood mononuclear cells of healthy donors (with their informed consent) were cultured for 72 h under different conditions of treatment with and without NLGP and/or LCA pulsation and their proliferation was assessed by MTT assay. ConA (Concanavalin A) was used as positive control. Error bars shown as standard error (SE) is obtained from three individual experiments (***p<0.001 for LCA+NLGP in comparison to NT and LCA).

NLGP displayed its ability to induce sufficient release of intramacrophage nitric oxide, which was higher than the normal concentration (*viz.* non-treated) that helped in the killing of tumor cells by NO directed cell death (Figure 2).

Type-1 immune response governs the release of nitric oxide from macrophages

To determine the immune response triggered by NLGP, the type of cytokines secreted was determined by the cytokine ELISA assay. The differentially pulsed macrophages with LCA antigen were co-cultured with lymphocytes, the concentrations of cytokines released in the cell-free culture supernatant were determined. Cytokine levels (Figure 3) of IFN-γ and IL-12 were found to be higher ($p<0.001$) IL-4 and IL-10 were found to be lower by NLGP treatment ($p<0.001$). Increased accumulation of NO was also involved in increased concentration of type-1 cytokines IFN-γ and IL-12, and a subsequent decrease in the concentration of type-2 cytokines IL-4 and IL-10 (Figure 3).

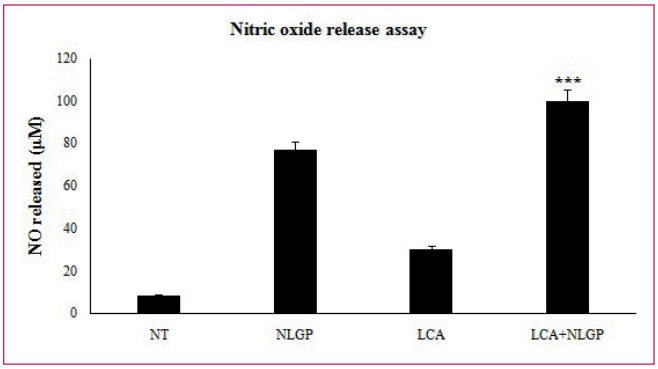

Figure 2. Intramacrophageal nitric oxide released in the culture media by macrophages under different conditions of treatment with and without NLGP and/or LCA pulsation were accessed by colorimetric assay based on Griess reaction. Data presented in the bar graph with standard error (SE) as error bars is of three independent experiments (***$p<0.001$ in comparison to LCA pulsed macrophages).

NLGP selectively increases the production of type-1 secretion of cytokines IFN-γ, IL-12 and not type-2 cytokines IL-4, IL-10

We investigated whether LCA+NLGP treatment creates type 1 immune environment and releases enhanced

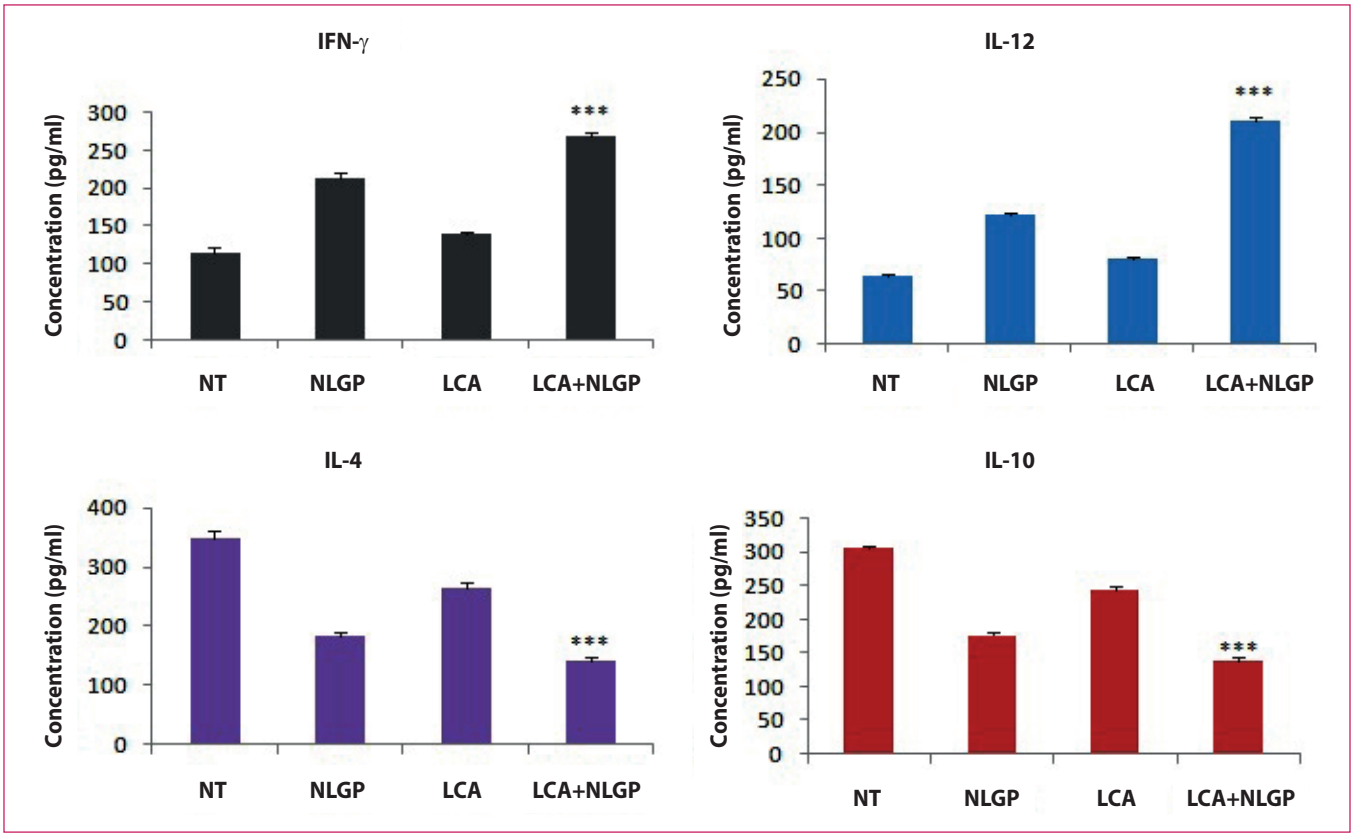

Figure 3. Assessment of cytokine secretory profile (for IFN-γ, IL-12, IL-10, and IL-4) after co-culture of differentially pulsed macrophages with autologous and allogenic treated lymphocytes was carried out. Supernatants were collected for cytokine ELISA after 48 h. NLGP enhances LCA specific secretion of Th1 cytokines (IFN-γ and IL-12) and reduces secretion of Th2 cytokines (IL-4 and IL-10). Error bars are standard error (SE) of three independent experiments (***$p<0.001$ for IFN-γ, IL-12, IL-4 and IL-10 in comparison to LCA).

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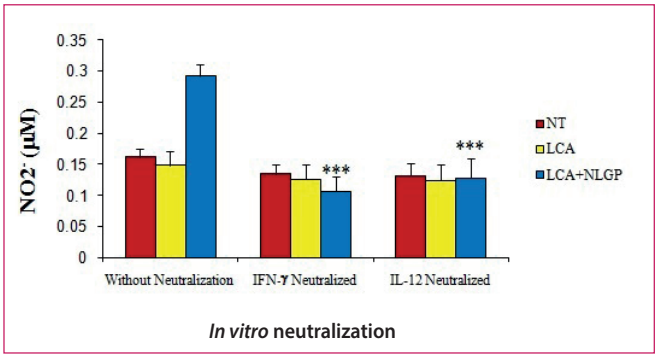
amount of NO from macrophages. To ascertain the dependence of two antitumor conditions produced during LCA+NLGP treatment, type 1 (IFN-γ, IL-12) and type 2 (IL-10, IL-4) cytokines were neutralized in macrophage/lymphocytes cell culture by using antibodies against specific cytokines. It was found that neutralization of IFN-γ and IL-12 in culture caused drastic decrease in NO release from macrophages (Figure 4a). This result suggests vaccination induced significant increase in IFN-γ and IL-12 release might play a pivotal role in triggering the signal for enhanced NO generation. On the other hand, neutralization of IL-10 and IL-4 resulted no change in NO content of macrophage culture supernatant (Figure 4b).

NLGP helps the macrophages to present LCA, which not only promotes proliferation of lymphocytes but also T-cell mediated cytotoxicity

To investigate the potential of NLGP as a mediator in eliminating lung cancer *in vitro,* A549 cell line was used. The differentially treated macrophages and lymphocytes were co-cultured with A549 cells, in the presence or absence of NLGP and LCA. The cytotoxic effects were determined with the LDH release assay. NLGP and LCA induced potent cytotoxic reponse (Figure 5).



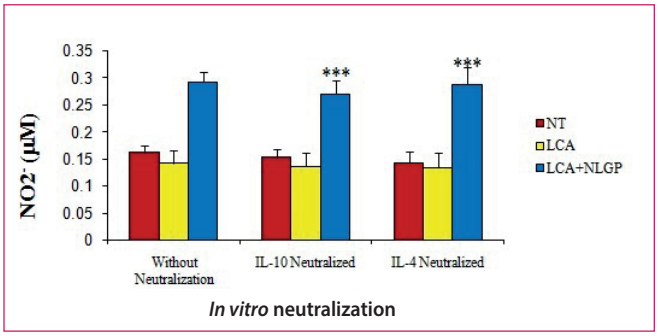


Figure 4. NO release dependence on Th-1 cytokine release was tested by neutralization assay. Type-1 cytokines (IFN-γ and IL-12) (a) and type-2 cytokines (IL-10 and IL-4) (b) were neutralized by their corresponding antibodies in macrophage treated with LCA and/or NLGP. After neutralization, the supernatants were collected after and were used to measure the NO using Griess reagent (***p<0.001 for IFN-γ, IL-12, IL-10 and IL-4). Each data represents mean values which were obtained from five individual experiments.

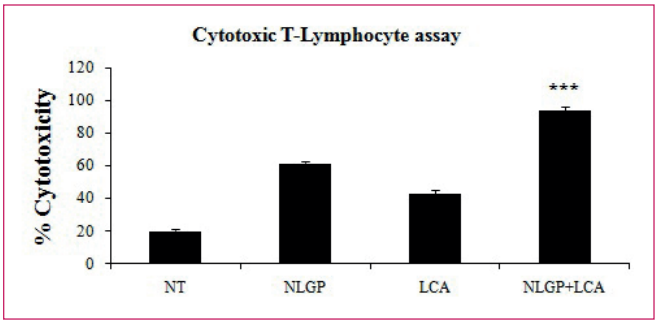


Figure 5. Cytotoxic T Lymphocytes (1×10^5) generated from co-culture of differentially macrophages with lymphocytes were incubated with A549 cells (1×10^4) overnight to obtain LCA specific cytotoxic lymphocytes. The CTL mediated cytotoxicity which was tested by LDH release assay using cytotoxicity detection kit. Data obtained from E: T (Effector cells: Target cells ratio) of 10:1 is presented Triton-X was used as positive control. Error bars are standard error (SE) of three independent experiments (***p<0.001 in comparison with LCA).

Discussion

Macrophage-mediated anti-tumor activity is dependent primarily on its activation by the antigen and an adjuvant that helps to further augment the immune response generated whilst activating specific T-cell subsets that target tumor growth and proliferation that helps to obliterate cancer cells through the cytotoxic T-lymphocytes.[7] The adjuvanticity and immunostimulating properties of non-toxic NLGP as reported earlier, has been successfully tested against LCA. It enabled the macrophages to present LCA more effectively to the T-cells.

In addition, NLGP simultaneously stimulated macrophages to produce nitric oxide. Nitric oxide, which is a highly reactive molecule is produced by inducible NO synthase enzyme *i.e.* (iNOS).[26] This highly reactive molecular species plays a dual role of cytotoxic and cytoprotective mechanism[26], therefore its production is greatly regulated and a dedicated study focused on the production NO at various levels demands further attention.

The intramacrophageal nitric oxide released by activated macrophages has been reported to eradicate tumor cells, promote apoptosis and also regulate the production of cytokines. This release of NO is primarily dependent on type-1 immune environment, favoring the releasing of

IFN-γ and IL-12, and downregulating the secretion of type-2 cytokines[7], IL-4, and IL-10 which support type-2 immune environment. Previously, NLGP was reported to hold the capability of being an inducer independently stimulating the T cells to release IFN-γ and macrophages to release IL-2.[27] Our current study chiefly based how neem leaf glycoprotein (NLGP) facilitates lung carcinoma-associated antigen (LCA)-specific anti-cancer immune response utilizing macrophage-mediated antigen presentation and induction of type 1 cytokines (IL-12, IFN-γ) coupled with nitric oxide production. We also analyzed whether LCA+NLGP treatment creates type 1 immune environment and releases enhanced amount of NO from macrophages. To ascertain the dependence of two antitumor conditions produced during LCA+NLGP treatment, type 1 (IFN-γ, IL-12) and type 2 (IL-10, IL-4) cytokines were neutralized in macrophage/lymphocytes cell culture by using antibodies against specific cytokines. It was found that neutralization of IFN-γ and IL-12 in culture caused drastic decrease in NO release from macrophages.[7] These results suggest that significant increase in IFN-γ and IL-12 release might play a pivotal role in triggering the signal for enhanced NO generation. On the other hand, neutralization of IL-10 and IL-4 resulted no change in NO content of macrophage culture supernatant.

As discussed earlier, in addition to IFN-γ and IL-12; IL-17 plays a pivotal role in the release of NO.[27–29] The release of IL-17 is governed by the Th17 cells in the milieu of cytokines such as TGFβ, IL-6, IL-23. Furthermore, cytokines IFN-γ, IL-4 which stimulate type-1 and type-2 immunity respectively have been shown to negatively regulate Th17 differentiation.[30] An increase in the proliferation of cytotoxic T lymphocytes as a result of type-1 immune system activation was also observed. The cytotoxic reactions, thus, enabled the elimination of tumor cells A549 *in vitro.*

However, our findings are limited to the *in vitro* study of NLGP assisted antigen presentation of LCA and the immune response generated, the further scope will be to carry out the study *in vivo* and also determine the cross-talk between LCA and macrophages and T-cells and its potential role in providing long-term immunity by creating memory B cells. In-depth study of the mechanism involved during the interaction of NO with IFN-γ, IL-12 and any aggressive course thereof, will be our future prospect for research.

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Indian Patent (No. 259434): "Process for isolating glycoprotein from neem leaf and its characterization to define the imunomodulatory and cancer preventive functions of this glycoprotein" was used for the current research.

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Peer-review: Externally peer-reviewed.

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Author Contributions: *Study design/planning:* Koustav SARKAR; *Data collection/entry:* Akanksha RAI†, Akshaya Mahalakshmi SURENDRAN†, Krithika Sonali K.NATARAJAN, Shreyosi BOSE, Shairee SANYAL, Sumantra MONDAL, Sayantanee MUKHERJEE; *Data analysis/statistics:* Akanksha RAI†, Akshaya Mahalakshmi SURENDRAN†; *Data interpretation:* Koustav Sarkar; *Manuscript preparation:* Koustav Sarkar; *Literature search/analysis:* Akanksha RAI†, Akshaya Mahalakshmi SURENDRAN†.

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