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Novel trisaccharide based phospholipids as immunomodulators

Srinivasa Reddy Bonam[a,b,c,1], Debabrata Bhunia[a,1], Sylviane Muller[c,d], Sridhar Goud Nerella[e], Mallika Alvala[e], Sampath Kumar Halmuthur Mahabalarao[a,b,*]

[a] Vaccine Immunology Laboratory, Natural Products Chemistry Division, CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad, 500007, India
[b] Academy of Scientific and Innovative Research (AcSIR), CSIR-IICT Campus, Hyderabad, 500007, India
[c] UMR 7242 CNRS- Neuroimmunology & Peptide Therapy Team, University of Strasbourg, Biotechnology and cell signaling, Illkirch, France/Laboratory of excellence Medalis, Institut de science et d'ingénierie supramoléculaire (ISIS), 67000, Strasbourg, France
[d] University of Strasbourg Institute for Advanced Study (USIAS), 67000 Strasbourg, France
[e] Molecular Modeling Facility, Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad, 500037, India

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ABSTRACT

A focused library of novel mannosylated glycophospholipids was synthesized employing imidate coupling and H-phosphate phosphorylation methods. All novel glycophospholipids were evaluated for their receptor interactions by molecular docking studies. Docking studies revealed dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) specific interaction of the glycophospholipid ligand **P4** acts, which was further confirmed by *in vitro* DC-SIGN expression on monocyte-derived dendritic cells (MoDCs). Further, *in vitro* and *in vivo* immunomodulatory activity among the six compounds (**P1**-**P6**) examined, compound **P4** displayed good immunopotentiation and adjuvant properties as indicated by the induced cytokine expression and enhanced ovalbumin (OVA) specific antibody (IgG) titers. Phosphatidylinositol mannosides (PIMs) analogues in the present study enforced the immunomodulatory properties, truncating parent PIMs or tailor-made of PIMs may bring the novel efficacious molecules, which will be useful in vaccine preparation against different diseases.

1. Introduction

Mycobacteria, fungus, and few other cell wall products exhibit powerful immunomodulatory properties and notably confer protection against atopic disorders. The greater part of the immune modulating components of mycobacteria is attributed to the presence of typical glycolipid signature on their cell wall, such as phosphatidylinositol mannosides (PIMs), lipomannans (LM), and lipoarabinomannan (LAM), a number of other *mycobacterial* components, which strongly modulate the immune responses [1,2]. Innate immune cells including dendritic cells (DCs), macrophages, natural killer (NK) cells, neutrophils, and monocytes efficiently recognize aforementioned cell wall components through a diversified pattern recognition receptors (PRRs), especially C-type lectin receptors (CLRs) [3]. Due to synergistic responses of immune stimulatory *mycobacterial* components with antigens, the formulations derived from such products have been exploited as vaccine adjuvants [4–6]. It is the case, for example of PIMs, which are glycolipids located on the *mycobacterial* cell wall that anchor an arrangement of complex glycolipids in their membranes [7,8]. It has been established that the smaller PIM molecules, retain immunomodulatory activities by themselves and especially stimulate cytokine production by activating immune cells that are key regulators of inflammatory cytokine expression in our immune system [9–12]. Thus, PIMs and their structural analogues, besides their role as antagonists [13] of atopic manifestations, also display unique properties as adjuvants enhancing the potency of sub-unit vaccines, with or without delivery systems [4,5]. It has been demonstrated that natural and/or synthetic glycolipid mimics of PIMs given either by the systemic or mucosal route catalyze cell-mediated immune responses. Structure-activity relationship (SAR) studies of PIMs have been conducted with several series of analogues of PIMs to discover potent immunomodulators, and few of them exhibited enhanced Th1 response (IL-12, *in vitro*, by murine DCs) [14]. Further, it was hypothesized that glycophospholipids like PIMs are potential inducers of the cell-mediated immune response, and a major challenge would be to design and synthesize well defined structural analogues and mimics that exhibit potential immunomodulation, with limited side effects.

Thus, the discovery of the mediator role of PIMs in the immune

* Corresponding author at: Vaccine Immunology Laboratory, Natural Products Chemistry Division, CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad, 500007, India.
E-mail address: sampath@iict.res.in (S.K. Halmuthur Mahabalarao).
[1] contributed equally and are co-first authors

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regulatory processes intensified the chemical construction of analogues of these compounds and many other related analogues. Recently, it has been discovered that a purified fraction of GPI from *Trypanosoma cruzi* trypomastigote mucins (tGPI) possessed tremendous pro-inflammatory activities, comparable to the bacterial lipopolysaccharide (LPS). By its ability to activate the macrophages to release tumor necrosis factor-*α* (TNF-*α*), interleukin-12 (IL-12), and nitric oxide (NO) production at the optimum level, tGPI represents one of the most potent immunomodulatory and pro-inflammatory inducing agents encountered [15,16].

Activation of immune cells, such as macrophages, DCs, and $CD4^+$ T cells with stimulatory molecules represents a highly relevant option for establishing the link between innate and adaptive immunity [17]. This strategy is very useful for targeting therapeutic interventions in certain diseases and immunocompromised conditions. In view of the promising adjuvant activity displayed by PIMs [18], we felt the need to explore the possibility of developing structural mimics of PIMs, encompassing a glucose unit sandwiched between two mannose residues, designed to target mannose receptors on DCs. The anomeric hydroxyl of glucose residue is conjugated to a branched lipid through a phosphate linkage as encountered in PIMs. Such novel designer glycophospholipids are expected to enhance the rapid internalization of the antigen by inducing endocytosis. The overall molecular framework resembles PIMs, wherein the inositol moiety is replaced with *α*-D-glucose moiety mannosylated at its 2,3-position through *α*-linkage. In the glycerol ester moiety, different long chain fatty acids are incorporated forming hydrophobic part of the glycophospholipids as shown in Fig. 1. In the present study, we describe the synthesis of a focused library of novel trisaccharide based glycophospholipids (See Supporting information Section 3) and their immunopharmacological evaluation using immune cell subsets, namely immune cells like macrophages, DCs, and $CD4^+$ T cells. The immunopharmacological data reported here provide insights into the SAR of novel glycophospholipid towards their molecular target preference.

2. Materials and methods

2.1. Cell lines and reagents

2.1.1. Mice

The study protocol related to animal use was approved by the Institutional Animal Ethics Committee (IAEC) of the Council of Scientific & Industrial Research-Indian Institute of Chemical Technology (CSIR-IICT) (IICT/BIO/TOX/PG/1/02/2013). BALB/c mice (female), weighing 25–28 g, 7–8 weeks old were obtained from the Center for Cellular and Molecular Biology (CCMB) Hyderabad, India and maintained under standard laboratory conditions (temperature 22 ± 2 °C, relative humidity 50 ± 15%, 12:12 light/dark cycle). All animals were given free access to water and food *ad libitum*.

2.1.2. Reagents

Cell culture materials like PBS, RPMI-1640 media (AT028), fetal bovine serum (FBS) (RM10409) and others were purchased from the Himedia (India). Cell surface markers, anti-CD3-allophycocyanin (APC)-Cy7 (557596), anti-CD19-phycoerythrin (PE) (553786), anti-CD4-fluorescein isothiocyanate (FITC) (553047), anti-CD8-PE (553033), anti-CD49b-PE (553858) and anti-T cell receptor beta (TCRβ)-APC (553174) antibodies were obtained from BD Biosciences (San Jose, CA, USA), anti-Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)-PE (FAB8345P) were purchased from R&D Systems, goat anti-mouse IgG, cytokines (IL-2 (purified; 503702, biotinylated; 503804), IL-4 (purified; 504102, biotinylated; 504202), IL-6 (purified; 504604, biotinylated; 504602), IL-12 (purified; 511802, biotinylated; 505302), and TNF-α (purified; 510802, biotinylated; 506312)) and recombinant Granulocyte-macrophage colony-stimulating factor (GM-CSF) (713604), IL-4 (714904) were procured from the BioLegend (San Diego, California) and goat anti-mouse IgG (sc-2005), goat anti-mouse IgG1 (sc-2060), goat anti-mouse IgG2a (sc-2061) from Southern Biotech (Birmingham, USA). Mouse Pan-DC enrichment Kit (19763), mouse $CD4^+$ T Cell Isolation Kit (19852) from Stemcell technologies (Vancouver, Canada). All reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Docking studies

Ligand docking is one of the major steps involved in drug discovery. This drastically reduces the time for identification of a lead molecule [19]. Several steps are involved in this process to run smoothly. All computational calculations were carried using 3D crystal co-ordinates of tlr2 (PDB ID:1FYW) apo protein from Protein databank. Active site pocket was determined using the sitemap module of schrodinger suite 2017. Based on the D-score and Hydrophobic/Hydrophilic scores, site 5 has been considered as potential binding sites and also from previous studies by Mistry P *et al.*, 2015, suggesting the presence Y647, C673, D678, F679, I680, K683, D687, N688, D691, and S692 amino acid residues in the binding sites pocket. Further flexible docking has been carried using Glide, version 6.1, (Schrödinger, LLC, New York, NY, 2013.Inc. 2012).

2.3. In vitro

2.3.1. Cytotoxicity

2.3.1.1. Splenocytes viability assay. Animals were sacrificed under light ether anesthesia. The lymphocytes from spleen were isolated aseptically in RPMI 1640 medium. Briefly, single cell suspensions were prepared by homogenization of spleen between the ends of frosted slides and homogenized cells were passed through 100 μM cell strainer. Cells were centrifuged at 400*g* for 10 min at 4 °C. Red blood cells (RBCs) were lysed with RBC Lysis buffer (0.5 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM disodium ethylene diamine tetra acetic acid, pH 7.2) for 5 min at 4 °C or 90 s at room temperature. Lymphocytes obtained were then washed twice with phosphate-buffered saline (PBS) and cell density was counted by the trypan blue dye exclusion method. Finally, spleen cell suspension (10^5 cell/well) was prepared in complete medium (RPMI 1640 media supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) FBS) and seeded into 96-well plates containing glycophospholipids and incubated for 48 h at 37 °C. After

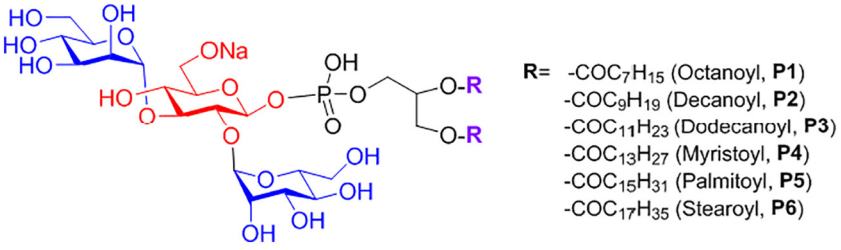

Fig. 1. Novel α-mannosylated glycophospholipids (**P1**-**P6**).

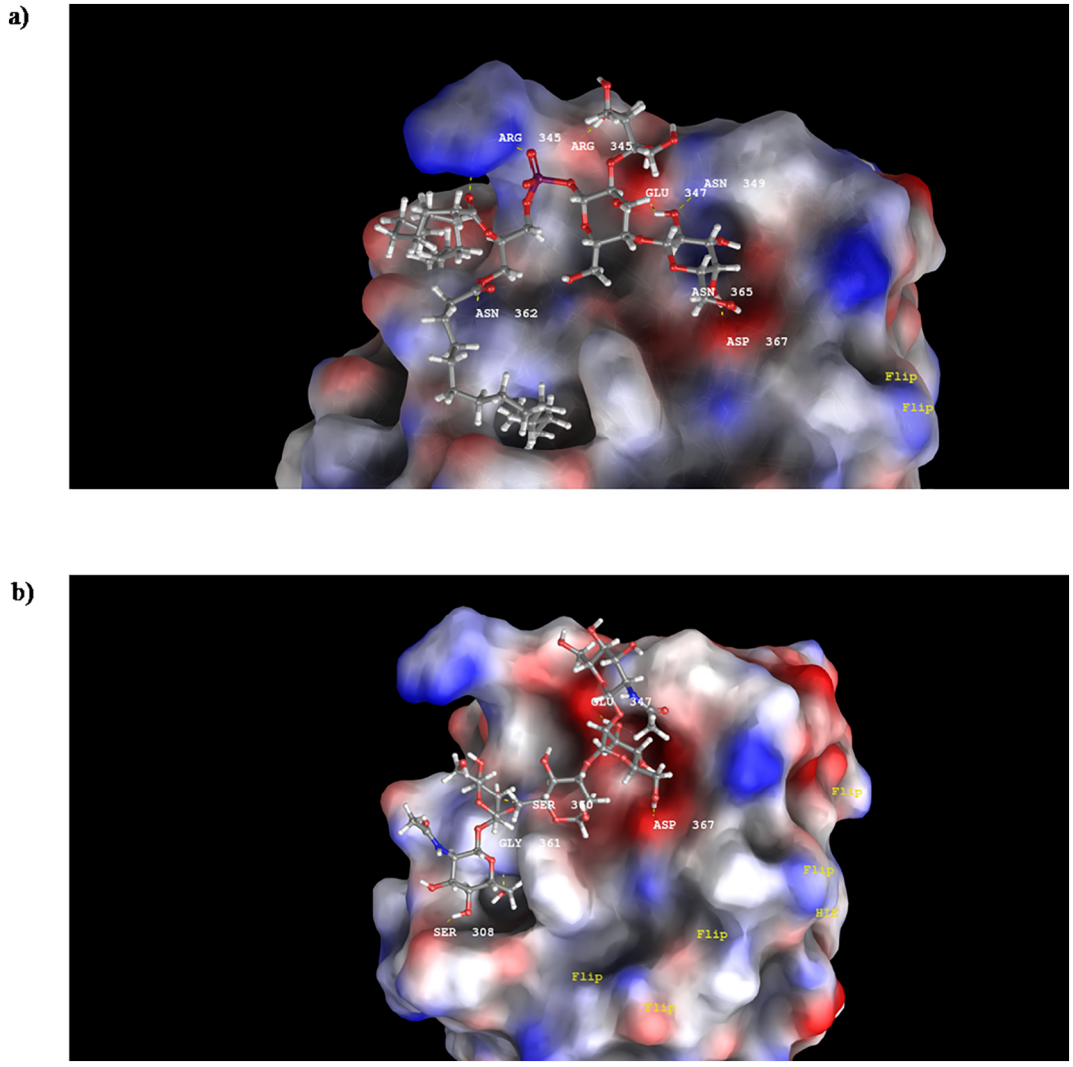

Fig. 2. The binding orientation of the glycolipids to DC-SIGN is shown. Surface view of the active site of DC-SIGN receptor, for the highest docking score confirmation. a. Myristic acid glycolipids. b. NAG-MAn-Man-Man-NAG repeats HBO and displayed in yellow dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Molecular docking results showing docking score and amino acid interactions.

Compound	Docking score	Amino acid interactions
P1	−8.412	Arg 345, Glu 354, Asp 367, Asn 362,
P2	−8.278	Glu 354, Asp 367, Asn 362, Glu 357
P3	−7.826	Arg 345, Glu 354, Asp 367, Asn 362,
P4	−9.017	Arg 345, Glu 354, Asp 367, Asn 362, Glu 357
P5	−7.278	Glu 354, Asp 367, Asn 362, Glu 357
P6	−6.488	Arg 345, Glu 354, Asp 367, Asn 362,
Co-Crystal (NAG-Man-Man-Man-NAG)	−6.154	Asp 367, Glu 354, Asn 365, Asn 349, Glu 347

incubation, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) (5 mg/mL in PBS) solution was added to each well and incubated for 4 h. The plates were centrifuged at 400*g* for 5 min and the supernatant was removed. To each well, 100 µL of a dimethyl sulfoxide (DMSO) solution was added and kept aside for 15 min and the absorbance was taken at 630 nm in TECAN multimode reader [20,21].

2.3.1.2. Human peripheral blood mononuclear cell (PBMCs) viability assay. PBMCs from whole blood (from a healthy human volunteer; CSIR-IICT-CCMB dispensary guidelines and volunteer consent note 03/2015) were separated by density gradient separation by using histopaque. PBMCs, 2×10^5 cells/well in 96-well plates were cultured for 24 h without or with glycophospholipids (50 µg/mL) or LPS (1 µg/mL). PBMCs viabilities were measured by using MTT method (see Section 2.3.1.1) [22].

2.3.2. Isolation and stimulation of mouse peritoneal macrophages

Peritoneal macrophages from naïve mouse were collected by using the method described by Tabasum *et al* (Supporting information 1.1). Macrophages 2×10^4 cells/well were seeded into 96-well plates and maintained overnight at 37 °C. After overnight incubation cells were

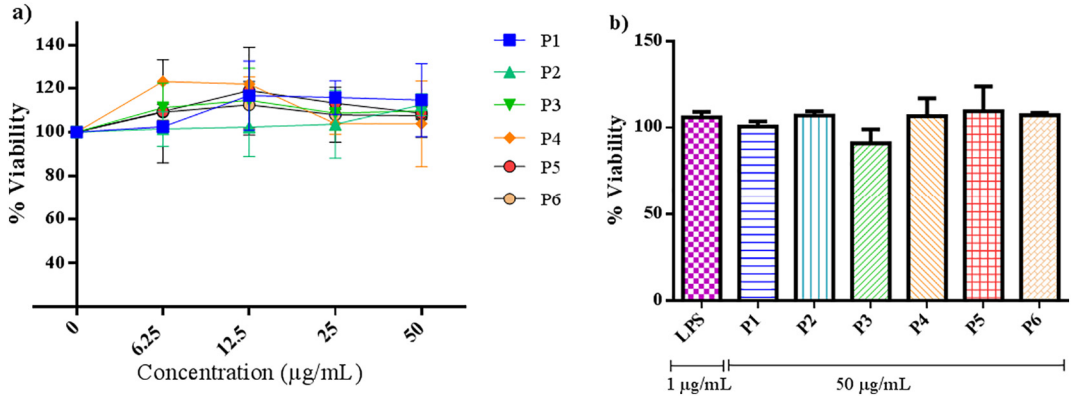

Fig. 3. Effect of glycophospholipid analogues on splenocytes and PBMCs viability. a. Splenocytes were prepared and cultured with glycophospholipid analogues at indicated concentrations for 48 h. b. PBMCs viability assay. Human PBMCs were separated by density gradient separation and treated with glycophospholipids. Splenocyte and PBMCs viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The % viability was measured and the values of mean ± SD (n = 3) of three independent experiments with triplicates are represented.

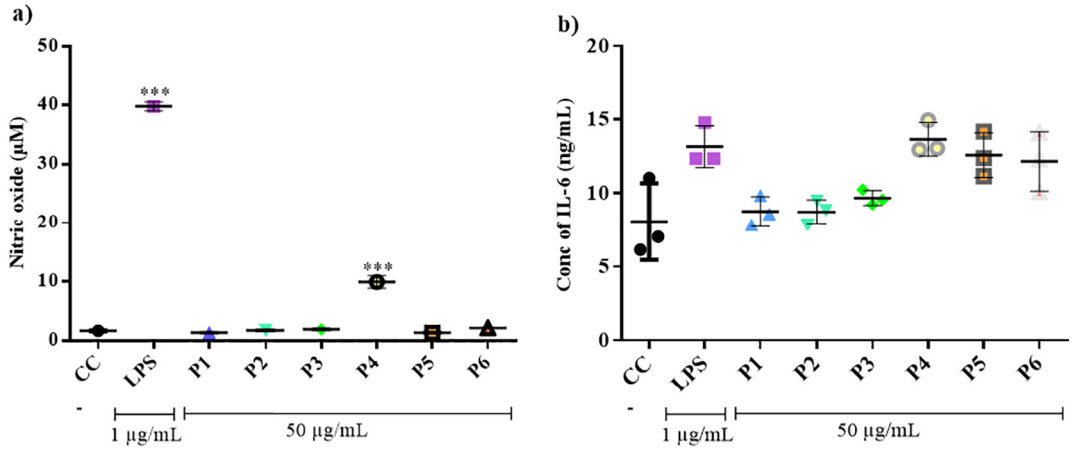

Fig. 4. Effect of glycophospholipid analogues on macrophages. Macrophages were stimulated without or with glycophospholipids for 16 h and supernatants were analyzed for nitrite content (Fig. 3a) by using the Griess reagent at 540 nm. In the same culture conditions, supernatants were analyzed for IL-6 (Fig. 3b) production by enzyme-linked immunosorbent assay (ELISA) method. The values represent mean ± SD of independent experiments with triplicate and significant differences are presented as $*P < 0.05$, $***P < 0.001$ *versus* cell control (untreated) (n = 3) (Dunnett's Multiple Comparison Test).

treated with test compounds at 50 µg/mL for 48 h and the supernatant was collected for the estimation of IL-6, IL-12, and NO (Supporting information 1.2) [23].

2.3.3. Endocytosis assay

Peritoneal macrophages were collected and seeded 2×10^4 cells/well in 96-well plate. Macrophages were treated with glycophospholipids at 50 µg/mL for 24 h. After 24 h of incubation, FITC conjugated ovalbumin (OVA) was added to the cells, endocytosis was left to proceed for 2 h at 37 °C and 5% CO_2. Thereafter, cells were washed with PBS twice and uptake of FITC conjugated OVA was quantified by fluorescence microscopy [24].

2.3.4. Isolation and stimulation of mouse DCs

DCs were isolated from the spleen of naïve BALB/c mice. Mouse Pan-DCs were separated from single cell suspensions by using EasySep™ Mouse Pan-DC Enrichment Kit. Separated cells were harvested and cultured in 6-well plates with GM-CSF and IL-4 10 ng/mL for 3 days. Further, 2×10^4 cells/well were seeded into 96-well plates and maintained overnight at 37 °C. After overnight incubation cells were treated with test compounds at 50 µg/mL for 48 h and the supernatant was collected for IL-12 estimation [25].

2.3.5. $CD4^+$ T cells stimulation assay

Naïve BALB/c mice splenocytes were used to separate $CD4^+$ T cells by using positive $CD4^+$ T cell selection kit (Supplementary Fig. 1). $CD4^+$ T cells (1×10^5 cells/well) were triggered in 96-well flat-bottomed micro titer plates. Soluble monoclonal antibodies (MAb), anti-CD28 1 µg/mL and anti-CD3 1 µg/mL were pre-coated in 96-wells plates. Cells were stimulated with novel glycophospholipids or LPS for 48 h. After 48 h incubation supernatants were analyzed for IL-2 and cells were used for viability assay [26,27].

2.3.6. In vitro antigen presentation assay

This assay was performed based on a method developed by Sprott *et al.*, with slight modification. DCs (10^5 cells/well) were plated in 96-well micro-titer plates and incubated overnight. Media was replaced with fresh media containing 10 µg of OVA. After 2 h incubation, test compounds (50 µg/mL) were added. After 4 h of incubation, the DCs were co-cultured with purified $CD4^+$ T cells (1×10^5 cells/well). The stimulation of glycophospholipids to $CD4^+$ T cells were quantified by IFN-γ produced in the supernatant cultures after 24 h [5,28].

2.3.7. In vitro immunophenotyping

Splenocytes were isolated from the naïve BALB/c mice. 1×10^6 cells/well in 6-well plates were cultured without or with glycophopholipids or LPS for 24 h. After incubation, cells were subjected

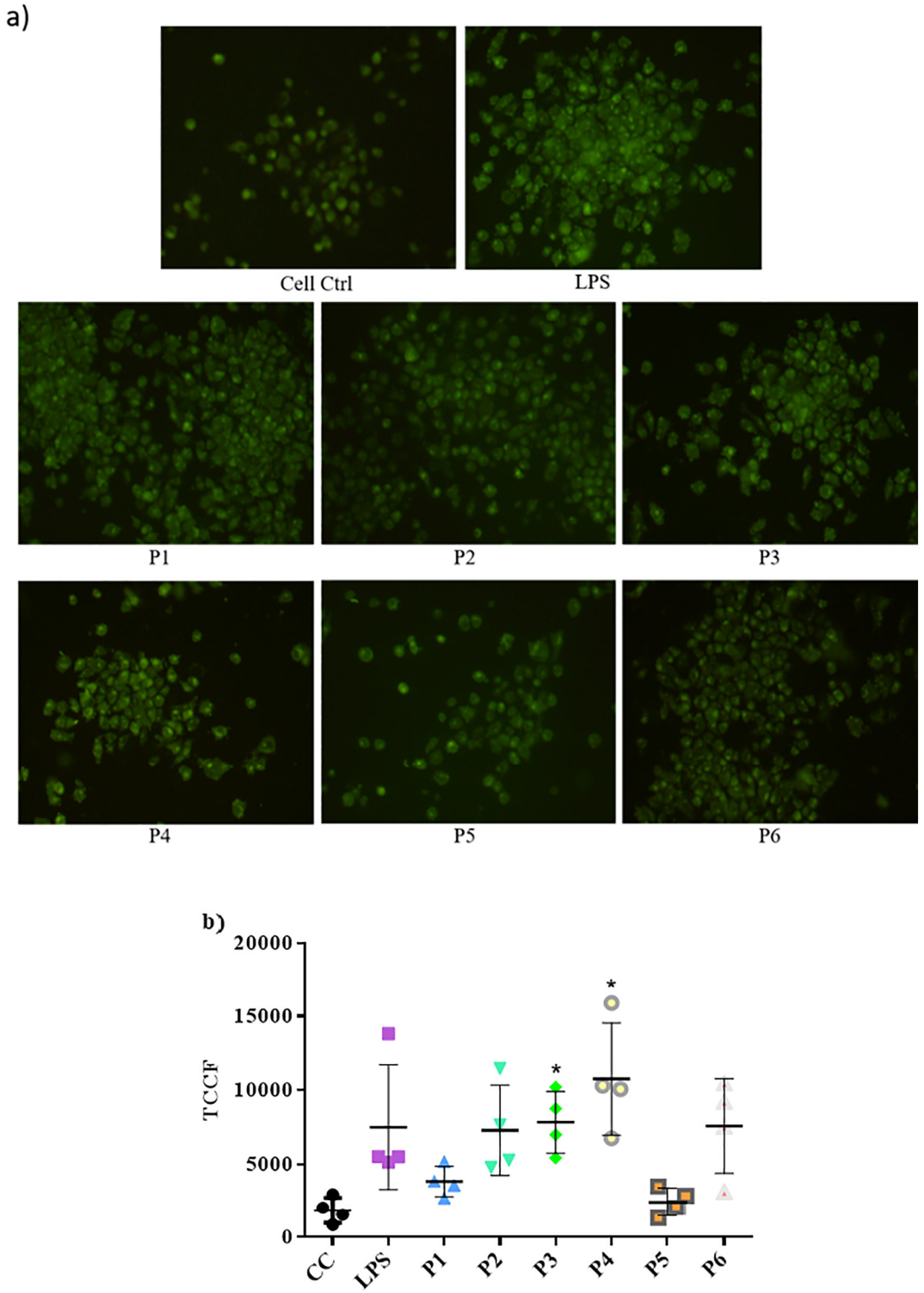

Fig. 5. Effect of glycophospholipids on endocytosis. a. Macrophages were stimulated without or with glycophospholipids (50 μg/mL) or LPS (1 μg/mL) for 24 h, and cells were incubated with FITC-conjugated OVA, for 2 h. The amount of conjugate uptake was assessed by fluorescence microscopy. b. Total fluorescence measurements were performed with Image J (v1.48, NIH), an outline was drawn in the region of each cell and the preset parameters *i.e.*, area, mean fluorescence, and integrated density measured, along with background readings. The fluorescence intensity bar graph was represented as total corrected cellular fluorescence (TCCF) (TCCF = integrated density – (area of selected cell × mean fluorescence of background readings)). The mean ± SD values of independent experiments are presented, and significant differences are represented as $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* cell control (conjugate controls) and $^{\#\#}P < 0.01$ *versus* LPS treated ($n = 4$) (Dunnett's Multiple Comparison Test).

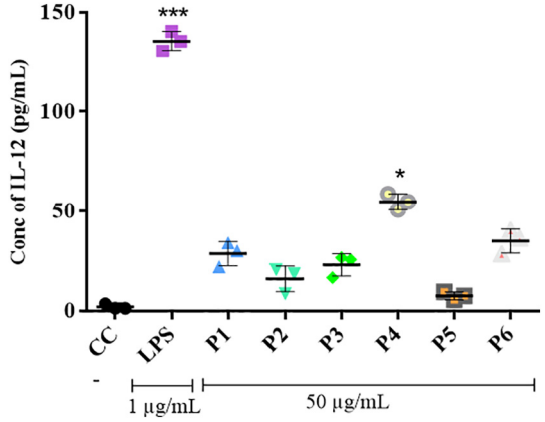

Fig. 6. DCs were stimulated with indicated glycophospholipid analogues, LPS or cell control for 48 h. Levels of IL-12 released by DCs were quantified by ELISA. The values are presented as mean ± SD of three independent experiments, and significant differences presented as $^{*}P < 0.05$, $^{***}P < 0.001$ *versus* cell control (untreated) (n = 3) (Dunnett's Multiple Comparison Test).

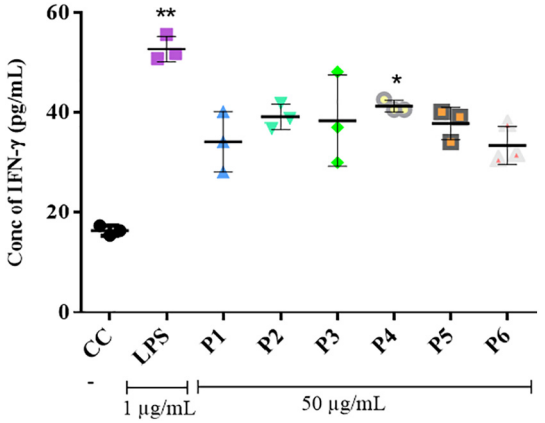

Fig. 8. *In vitro* antigen presentation assay. DCs were treated with OVA along with glycophospholipids for 16 h and $CD4^+$ T cells were co-cultured for further 24 h. The amount of IFN-γ release was quantified by ELISA. The values are presented as mean ± SD of three independent experiments, and significant differences presented as $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* cell control (untreated) (n = 3) (Dunnett's Multiple Comparison Test).

to centrifugation at 300*g* for 5 min, 4 °C. Anti-mouse antibodies such as anti-CD3-APC Cy7, anti-CD19-PE, anti-CD49b-PE, and anti-TCRβ-APC were used to stain spleen cells [29]. Data were collected by using flow cytometer (BD FACSVerse Fullerton, CA, USA) and analyzed using BD FACSuite™ software.

2.3.8. *In vitro DC-SIGN assay*

DCs were isolated from the spleen of naïve BALB/c mice. Mouse Pan-DCs were separated from single cell suspensions by using EasySep™ Mouse Pan-DC Enrichment Kit. Separated cells were harvested and cultured in 6-well plates with GM-CSF and IL-4 10 ng/mL for 3 days. Further, 3×10^5 cells/well were seeded into 24-well plates and cells were treated without or with LPS and/or **P4** at indicated doses for 48 h [25]. After incubation, cells were harvested and processed for PE-DC-SIGN staining. Data were collected by using flow cytometer (BD FACSVerse Fullerton, CA, USA) and analyzed using BD FACSuite™ software.

2.4. *In vivo adjuvant activity*

2.4.1. *Immunization*

Female mice (6–8 weeks) were divided into six groups, each consisting of five mice. Animals were subcutaneously (s.c.) immunized with OVA 100 µg alone and OVA 100 µg dissolved in saline containing *Quillaja saponaria* (QS)-21 (20 µg), **P4** (1, 10, and 100 µg) on day 1. Booster was given after 14 days of primary immunization. Sera and splenocytes were collected two weeks after the booster dose.

2.4.2. *Estimation of OVA-specific antibody titer*

Sera was collected from the retro-orbital plexus of the control and treated groups on the 28th day and subjected to estimation of OVA-specific IgG, IgG1, and IgG2a antibodies by indirect ELISA (Supporting information 1.3) [30,31].

2.4.3. *Splenocyte proliferation*

Splenocytes from immunized mice groups were aseptically removed and minced. Lymphocytes were obtained by lysing the RBCs and resulting suspension cells were incubated separately at 1×10^5 cell/well in 96-well microtiter plates using an RPMI 1640 medium with 10% (v/v) FBS along with 2 µg/mL Con A or 10 µg/mL LPS or 10 µg/mL OVA for 48 h at 37 °C in 5% CO_2. After incubation cell, viability was measured using the MTT assay [32].

2.4.4. *Immunophenotyping*

Lymphocytes isolated in the above procedure were used for immunophenotyping. The slightly modified procedure of Colovai A *et al.*, was used. 5×10^5 cells from all treated groups were taken into the 5 mL

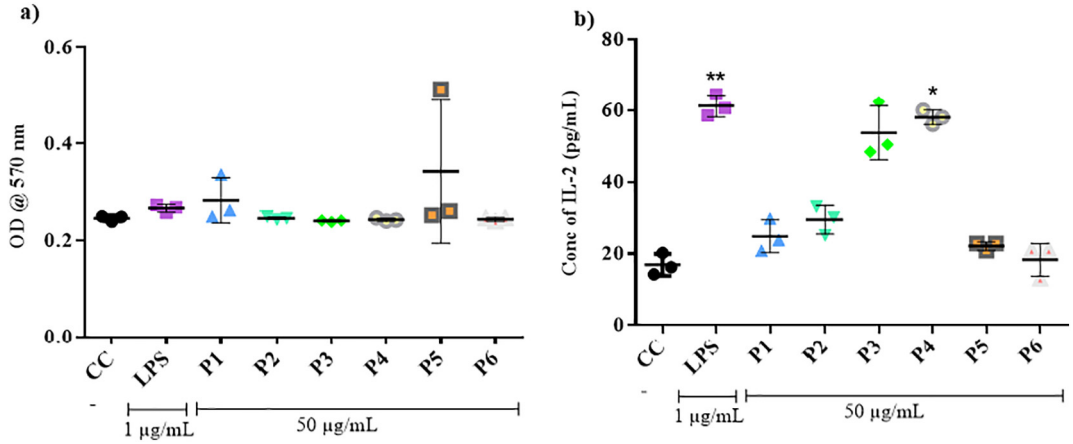

Fig. 7. $CD4^+$ T cell viability assay. Magnetically separated $CD4^+$ T cells were cultured without or with glycophopholipids or LPS for 24 h. a. Viability was done by using MTT assay. b. IL-2 quantification was done by ELISA. The values are presented as mean ± SD of two independent experiments, and significant differences presented as $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* cell control (untreated) ($n = 2$) (Dunnett's Multiple Comparison Test).

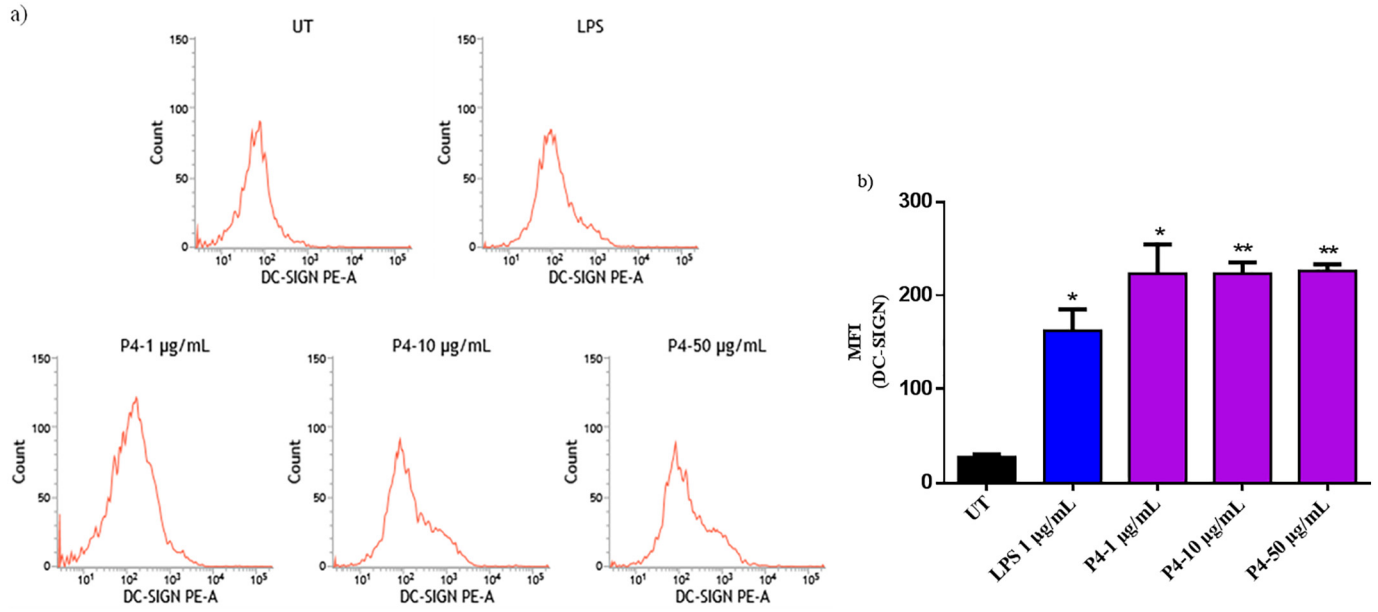

Fig. 9. DC-SIGN expression. Monocyte-derived mature DCs were treated without and with **P4** at indicated doses for 48 h. DC-SIGN expression was observed by using flow cytometry. a. Representative histograms were obtained by suspending the cells in a sheath buffer and collecting 100,000 cells by flow cytometry. b. Values from each treatment tested for mean fluorescence intensity (MFI) of DC-SIGN expression. The values represent mean ± SD of two independent experiments, and significant differences are presented as $*P < 0.05$, $**P < 0.01$ *versus* untreated (UT; mature DCs without treatment) (n = 2) (unpaired *t*-test).

polystyrene tube (BD Falcon™ round-bottom tubes). 1 μL of Fc (fragment crystallizable) block was added to the cells and incubated for 15 min. After incubation, the cells were harvested and centrifuged at 300*g* for 5 min at 4 °C. Saturating amounts of anti-CD4-FITC and anti-CD8-PE and conjugated antibodies were added to the cells and incubated for 45 min. After incubation, the unbound fluorochrome was washed away and suspended the cells in the sheath fluid for FACS analysis [29]. Data were collected by using flow cytometer (BD FACSVerse Fullerton, CA, USA) and analyzed using BD FACSuite™ software.

2.4.5. Cytokine estimation

Lymphocytes 1×10^5 cells/well were cultured in 96-well plates without or with OVA. After 48 h incubation, culture supernatants were subjected to measure cytokines by ELISA [33].

2.5. Statistical analysis

The data were analyzed using Prism software (GraphPad, San Diego, CA, USA). Data were expressed as mean ± SD and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test for pooled data or Dunnett's multiple comparison test for individual data) or unpaired *t*-test with (Mann–Whitney *U* test).

3. Results

3.1. SAR studies

In the present study, the focused library of PIMs structural mimics has been achieved by i). Swapping the inositol unit of PIMs scaffold with a D-glucose unit to examine the effect on the immunopharmacological attributes ii) varying the chain length of lipid moiety attached to sugar framework *via* phosphate linker to study the effect on the biological activity. Upon *in-silico* docking of various analogues (**P1**-**P6**) with target DC-SIGN protein, glycophospholipid containing myristic acid (**P4**) substitution has shown the highest binding affinity with a docking score of −9.017 compared to −6.154 of standard *N*-acetyl glucosamine-Mannose repeat (NAG-Man-Man-Man-NAG) co-crystal, which contributed to the strong hydrogen bond interactions with basic amine moiety of -Arg 345, acid side chain of -Glu 354, -Asp 367, and -Glu 357 along with amide side chain of Asn −362, within 3.00 Å distance (Fig. 2). Substitution with other fatty acids like octanoic (**P1**), decanoic (**P2**) and dodecaonic (**P3**) has shown moderate docking score whereas substitution with stearic acid (**P6**) and palmitic acid (**P5**) (more unsaturation) exhibited docking score which is almost equal to co-crystal. Docking scores and interacting amino acids within 5 Å distance were shown in Table 1.

3.2. Immunopharmacological activity

3.2.1. Determination of cytotoxicity of glycophospholipids on murine splenocytes and human PBMCs

The toxicity profile of glycophopholipids was examined by using MTT reduction cytotoxicity assay. Murine splenocytes were incubated for 48 h with or without glycophospholipids from 6.25 μg/mL to 50 μg/mL. Untreated cells (CC) were considered as control. As shown in Fig. 3a, no toxicity/cell death was detected up to 50 μg/mL concentration of glycophopholipid analogues **P1** to **P6**.

To determine the reactivity of glycophospholipids on the human cells, PBMCs were treated with glycophospholipids. As also observed with LPS, little or no significant proliferation was measured *ex vivo* compared to non-stimulated controls. This experiment was important to conclude that none of the glycophospholipids were toxic to the human PBMCs (Fig. 3b) [22].

3.2.2. Immunomodulatory properties of glycophospholipids on macrophages

Peritoneal macrophages treated with glycophospholipids **P1** to **P6**, or LPS, revealed that among the novel glycophospholipids studied here, **P4** was the most potent inducer of NO, although less efficiently than LPS (Fig. 4a). To characterize the possible effect of glycophospholipids on the pro-inflammatory response, macrophages were treated with glycophospholipids and the level of pro-inflammatory cytokines was measured in cell culture supernatant. The results indicate that **P4**, **P5**, and **P6** induced an increased release of IL-6, the level of which was comparable to that induced by LPS (Fig. 4b). Interestingly, the six glycophospholipids we designed and analyzed, only two of the compounds (not all), **P4** and **P5** appear to have induced significant levels of

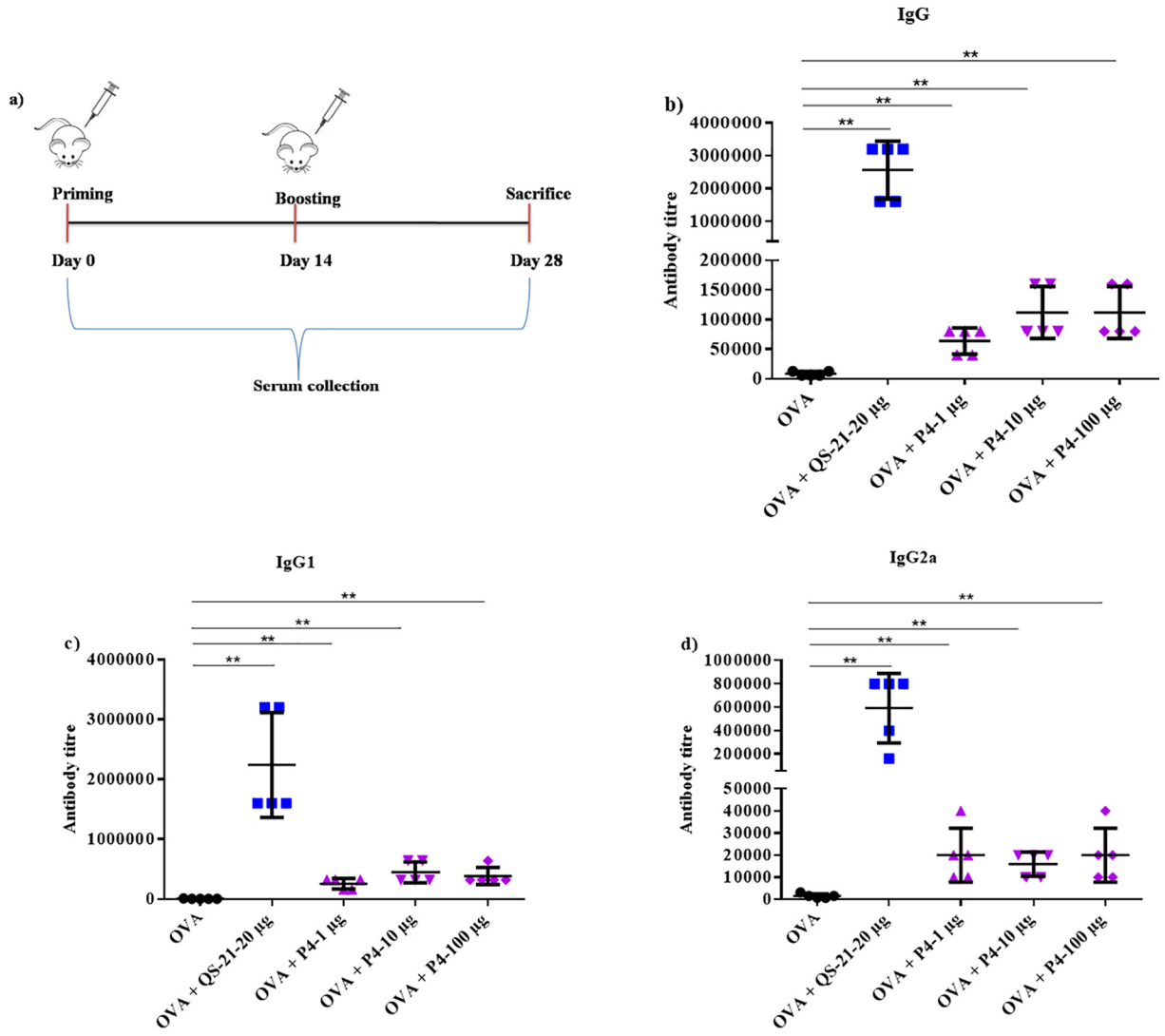

Fig. 10. Humoral immune response. (a) Schematic illustration of the immunization protocol used. Effect of glycophospholipds on OVA-specific IgG (b), IgG1 (c), and IgG2a (d) antibody. Mice were divided into different groups as mentioned in the methods and immunized subcutaneously. 14th day after booster dose, sera were collected from mice blood and antibodies were measured by an indirect ELISA (for procedure see Supporting information). The values are presented as mean ± SD and significant differences presented as $***P < 0.001$ *versus* OVA alone treated group ($n = 5$) (Mann–Whitney *U* test).

IL-12 in macrophages, as shown in the supplementary fig. 3.

3.2.3. Macrophage endocytosis

To evaluate the effect of novel glycophospholipids on endocytosis, mouse peritoneal macrophages were treated with glycophospholipids **P1** to **P6**, or LPS for 24 h. The activation of endocytosis in macrophages was confirmed by using FITC conjugated OVA, for 2 h. Events, such as antigen uptake, processing, and presentation to T-cells are controlled by innate immune system, which is critical for host primary immune defense. In this progression, pathogen-derived molecules or their mimics can influence profoundly the antigen presentation by the macrophages to T cells, allowing the induction of acquired immunity [34]. In this context, most of the analogues displayed endocytic activity except **P1** and **P5**. Indeed, compound **P4**, showed higher endocytic activity than LPS (Fig. 5a & b).

3.2.4. In vitro immune stimulatory experiments

Here, we show that IL-12p70 is effectively released from DCs in response to **P4** treatment although less efficiently than LPS, thus confirming that **P4** induces inflammatory components and directly stimulates DCs (Fig. 6). The effect of compounds **P1** to **P6** on T cells ($CD3^+$-Allophycocyanin Cy7 (APC Cy7)), T cells expressing T cell receptor beta chain ($TCR\beta^+$ - APC), NK ($CD49b^+$ - PE), and natural killer T (NKT) cells ($CD49b^+$ - PE $TCR\beta^+$- APC) as well as on B cells ($CD19^+$ - phycoerythrin (PE)) in heterogeneous spleanocytes was investigated by using flow cytometry (Supplementary Fig. 4). Compared to LPS-treated or untreated cells, no effect was shown in B cell population. However, the effects of our analogues on T cell populations were comparable and **P4** was even superior, indicating that the novel glycophospholipids activate the T cells. Our studies further showed that the NK cell population was not altered, on the contrary, the NKT cell population was affected and even more than in the presence of LPS (Supplementary Fig. 4).

Purified $CD4^+$ T cells were triggered for 16 h with monoclonal antibodies (anti-CD3 and anti-CD28) in the presence of OVA and glycophospholipids. $CD4^+$ T cell activation was confirmed by IL-2 production. Pure cultures of $CD4^+$ T cells incubated in the presence of glycophospholipids or LPS, did not show any significant proliferation or toxicity (Fig. 7a). Interestingly, however, raised IL-2 levels could be detected in the cultures of cells incubated with compounds **P3** and **P4** compared to cell control, in the magnitude similar to those secreted by $CD4^+$ T cells incubated with LPS (Fig. 7b). We tested

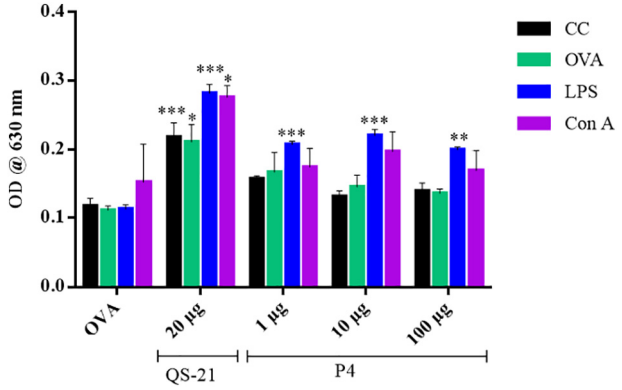

Fig. 11. Effect of glycophospholipids on splenocyte proliferation. Spleens were isolated (Supplementary Fig. 1) on the 28th day after primary and booster immunization, splenocytes were cultured with Con-A, LPS, OVA or media alone (CC) for 48 h. Splenocyte viability was measured by the MTT method as described in the methods. The values are presented as mean ± SD of absorbance and significant differences presented as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* OVA alone treated group (n = 5; pooled data) (Bonferroni's Multiple Comparison Test).

glycophospholipids effect on $CD4^+$ T cells in co-culture with DCs and OVA, as experimental antigen. In this assay, interestingly, all glycolipids appear to have an effect (release of IFN-γ), again **P4** was the most potent analogue, although less effective than LPS used in parallel (Fig. 8).

3.3. Effect of P4 on DC-SIGN expression

Monocyte-derived dendritic cells (MoDCs) were cultured without or with LPS and **P4** at indicated concentrations for 48 h. After incubation, cells were analyzed for DC-SIGN expression, which is generally expressed in the infected/inflammation/inflammatory signals condition [35]. This study revealed that, **P4** inducing increased expression of DC-SIGN, which is superior to LPS treatment (Fig. 9). However, the dose dependency of DC-SIGN expression was not observed in the **P4** treatment.

3.4. Adjuvant activity

A potent adjuvant produces a synergistic immune response against antigens, which is most desirable. To study the effect of glycophospholipid **P4** on antibody secreting functions of B cells, we used an indirect ELISA method to quantify the antibody response produced *in vivo*. As shown in Fig. 10a, after administration of an antigen (OVA) mixed with **P4** (Fig. 10b), antibodies (IgG) to OVA reached significant levels in **P4** treated the group as compared with the group of mice that received OVA alone, even though they were less significant in comparison with the QS-21 treated group. Moreover, a similar pattern was observed in the subtype analysis *i.e.*, IgG1 and IgG2a (Fig. 10c & d). A dose-effect was found indicating the range of **P4** efficacy (Fig. 10b-d).

We also evaluated the cellular immune responses to understand the T or B cell-mediated immunity to OVA in mice that received this immunogen mixed with **P4**. It is well known that Con-A and LPS stimulate T cells and B cells proliferation, respectively. These molecules were used as control. Fig. 11 depicts that QS-21 significantly enhanced the antigen and mitogen-induced spleen cell proliferation whereas **P4**, regardless of doses, significantly promoted the LPS induced proliferation as compared to the OVA-alone treated group. This indicates that **P4** directly or indirectly activated B cells *in vivo*.

More specifically, to evaluate the potential effects of the glycophospholipids on T cell subsets, we conducted an experiment using anti-CD4-FITC and anti-CD8-PE antibodies for T cell staining. Results revealed a significant increase in the $CD4^+$ and $CD8^+$ populations were observed in QS-21 (20 µg)-OVA treated group and **P4** (10 µg)-OVA treated group as compared to OVA-alone treated group (Fig. 12).

Th1 and Th2 cytokines were measured from splenocytes cultured in *ex vivo* conditions with OVA re-stimulation. These results indicate that secretion of Th1 cytokines, such as IL-2, IFN-γ, and IL-12 stimulates the production of IgG2a. On the other side production of IgG1 is depended on the Th2 cytokine, IL-4 secretion (Fig. 13). Whereas no significant expression of IFN-γ was observed in all compounds treated with **P4** compared with QS-21. However, interestingly increased expression of IL-12 was observed in all the treated groups as compared to OVA and QS-21-treated groups. Moreover, cytokine measurement from re-stimulated splenocyte cultures showed increased expression of IL-2 and IL-4 in a higher dose of **P4**-OVA treated group and QS-21-OVA treated group.

4. Discussion

Because of their wide immune stimulating properties, glycophospholipids are reported as potential adjuvants for vaccine development [6]. We, therefore, investigated *in vitro* immune stimulatory potential of these novel mannosylated synthetic glycophospholipids (Fig. 1) compared to LPS and explored their possible application as adjuvants through *in vivo* experiments on murine model in comparison with the standard glycolipid adjuvant QS-21.

The glycans from various pathogens including human immunodeficiency virus (HIV), Ebola virus and Hepatitis C virus, *Mycobacterium tuberculosis*, *Candida albicans*, and *Leishmania* parasites are primarily recognized by the C-type carbohydrate-recognition domains (CRDs) of DC-SIGN protein in the host system which evoked interest in us to study the targeting of DC-SIGN protein by synthesized molecules. It has been reported that in particular pathogens are targeted to the DC-SIGN (also known as CD209) for their internalization and possibly antigen presentation [36]. Pathogens interact DC-SIGN either to infect or to change the response from a Th1 to Th2-type of immunity, to favor their survival. Geijtenbeek *et al.*, demonstrated that *Mycobacterium tuberculosis* containing high mannose capped cell wall shows decreased co-stimulatory molecular expression thereby decreasing adaptive immunity, which is required for *mycobacterium* survival in DC and macrophages [37]. Co-culture assay shown in Fig. 8 clearly demonstrates the release of IFN-γ supporting the effect of **P4** on Th1 oriented immunity, which is essential to kill the intracellular pathogens (Fig. 13). These data led us to investigate whether **P4** could interact and/or stimulate DC-SIGN receptor. Molecular docking screening of synthetic glycophospholipids effectively revealed the binding and interaction of these ligands with receptor DC-SIGN (PDB ID: 1K9I) (Table 1). Among all, the glycophospholipids having myristoyl moiety has shown high docking score of −9.017 than other glycolipids (Fig. 1, Table 1). Based on the above docking data, we have additionally carried out the *in vitro* MoDCs stimulation with different doses of **P4** with or without LPS co-stimulation. As anticipated, by docking studies, **P4** has shown significantly increased expression of DC-SIGN (Fig. 9 & Supplementary Fig. 5), which is comparable to LPS. Innumerable literature discusses the cross-talk between toll-like receptors (TLR) and CLRs, especially DC-SIGN; it modulates and/or interact with the TLRs signals, including TLR2, TLR3, TLR4, NOD2, and others [37–41]. Considering the above vital information, we have co-treated the MoDCs with LPS (as a TLR4 agonist) along with **P4** and found that **P4** is exhibiting a synergistic effect with LPS. However, we did not observe the dose dependency (Supplementary Fig. 6). Further, we extended our docking study towards TLR2 receptor. Interestingly, in-silico results found that **P4** is also exhibiting TLR2 binding activity with a docking score of −4.166, but in comparison to DC-SIGN interaction, it is less (Supplementary Fig. 6) [38,41]. Altogether the data indicate that **P4** plays a major immunomodulatory role *via* involving DC-SIGN receptor, as demonstrated by *in vitro* results.

The previous study by Ainge *et al.*, with PIMs ether has given a clue

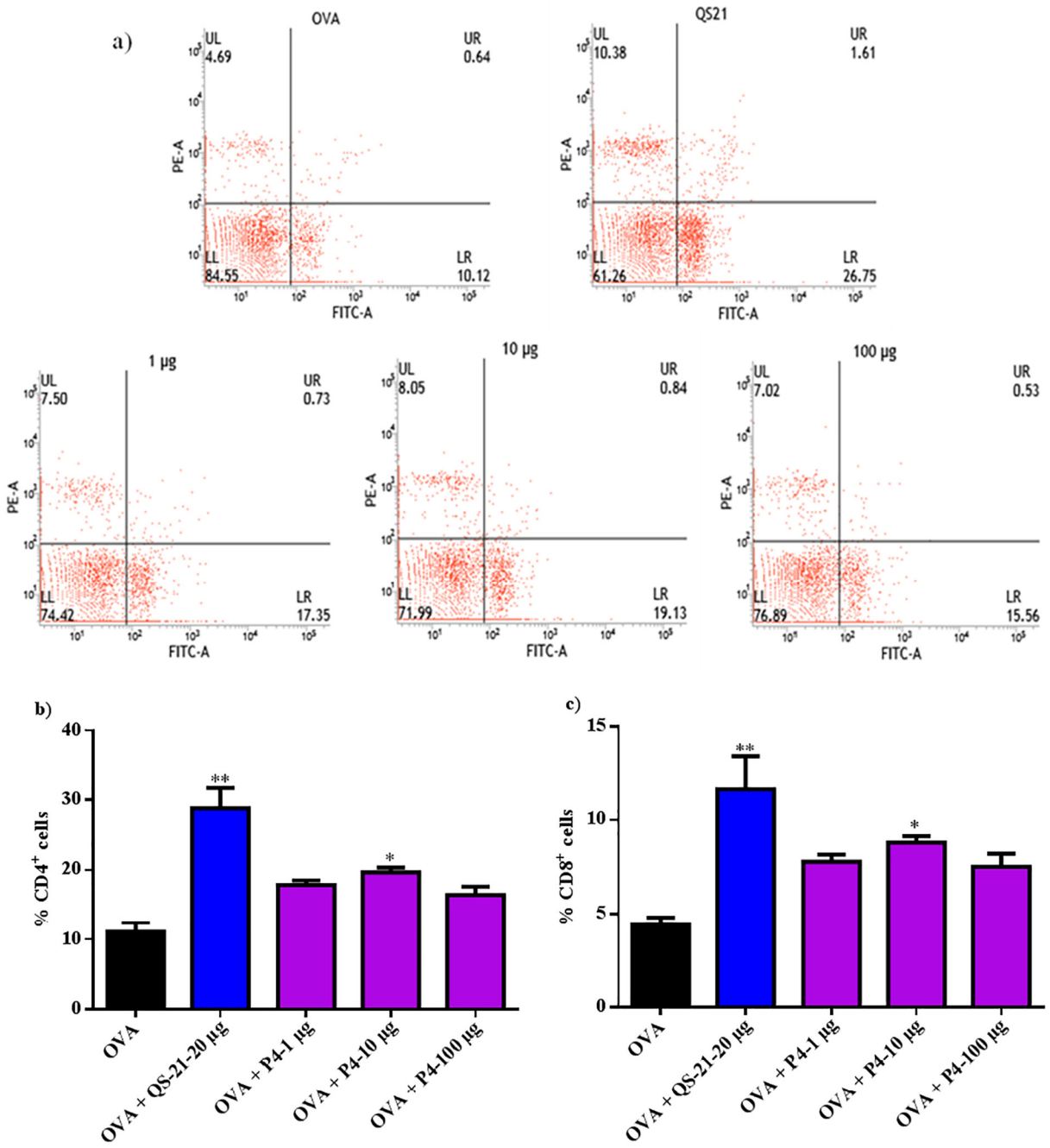

Fig. 12. Immunophenotyping by flow-cytometry. Spleen cells from indicated groups were stained with anti-CD4-FITC and anti-CD8-PE antibodies. a. Representative dot plots were obtained by suspending the cells in a sheath buffer and collecting 100,000 cells by flow cytometry. b. Values from each group tested for absolute numbers of $CD4^+$ (b) and $CD8^+$ (c) cell populations, in the spleen obtained from BALB/c mice. The values are presented as mean ± SD and significant differences presented as $^*P < 0.05$, $^{**}P < 0.01$ *versus* OVA alone treated group (n = 5; pooled data) (Bonferroni's Multiple Comparison Test).

for the selection of dose in immunological evaluation [42] and the same dose was carried out for all the *in vitro* experiments. Altogether these data indicate that **P4** which display non-toxic effect on murine and human immune cell (PBMCs) in *in vitro* conditions (Fig. 3). Brightbill *et al.*, reported that the *Borellia Ospa* and *M. tuberculosis* lipoproteins could activate a gene under the control of the iNOS promoter in macrophage cells [43]. Based on this clue, as our glycophospholipids resemble structural mimics of phosphatidylinositol mannosides, we subsequently tested the cellular response induced by these glycophospholipids or LPS on peritoneal macrophages. Production of NO from macrophages indicates the genesis of danger signals (IL-6 and IL-12) and this signal is essential for host defense against intracellular pathogens [44]. Among the compounds **P4**, **P5**, and **P6** have moderately stimulated the danger signals. However, NO is secreted only by stimulation with compound **P4** but no other glycophospholipids (Fig. 4). The release of pro-inflammatory cytokines from the macrophages upon stimulation with mannose and mannose containing analogues also indicates the alert signal from the immune system [45]. Clearly, novel mannosides or pseudo-mannosides induce the release of cytokine, chemokines, and they also activate the co-stimulatory molecules, which qualifies these molecules as an attractive alternative to the TLR based immunomodulators [46,47]. IL-12 possesses a wide variety of immunological properties including its ability to up-regulate Th1 type of cytokines (*e.g.* IFN-γ) and down-regulate Th2 type cytokines (*e.g.* IL-4). Moreover, it also enhances the cytotoxicity of NK and T cells along with the increased endocytic activity of macrophages [48]. Along

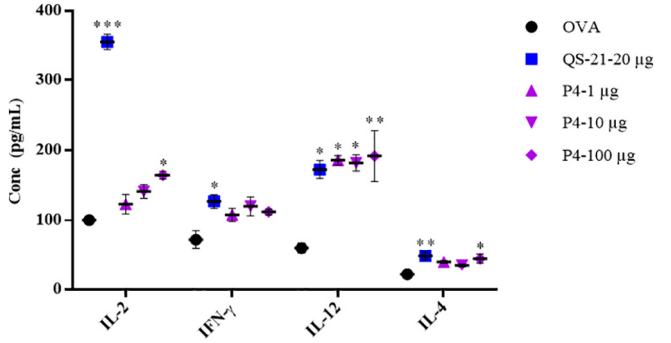

Fig. 13. *Ex vivo* cytokine production by splenocytes. Spleen cells were harvested from the different groups and re-stimulated with OVA for 48 h. After 48 h of incubation, culture supernatants were subjected for IL-2, IL-4, IL-12, and IFN-γ estimation. The values are presented as mean ± SD and significant differences presented as $*P < 0.05$, $**P < 0.01$, $**P < 0.001$ *versus* OVA alone treated group (n = 5; pooled data) (Bonferroni's Multiple Comparison Test).

with DCs, macrophages also capture, process, and present pathogens to the T cells, thus they regulate collaboration between innate and adaptive immune system. In consequence, it leads to the production of cytokines, chemokines, other immune signals (NO) and also recruitment and activation of other bystander cells [49]. CLRs are responsible for the endocytosis of the several fungal components in macrophages [50]. In the present study, we have used OVA as a model antigen. It has been proved that OVA is selectively up-taken by the mannose receptors [51,52]. Fig. 5 indicates the effect of glycophospholipids on macrophages for inducing alter signals in endocytosis process, which indirectly reveals the mannose receptor activation. PIMs were also shown to contribute to receptor-mediated internalization of *mycobacteria* in macrophages [53]. Mannose receptor activation not only mediates endocytosis, but it also controls the downstream signaling pathway [54]. Endocytosis assay is providing support for the potent IL-12 response observed in macrophage stimulation experiment discussed earlier. IL-12 response upon endocytosis represents the innate signaling mechanism of these glycophospholipids, which is essential against intracellular pathogens [55]. Together with the findings described above on cytokines, and these results strongly suggest that compound **P4**, which appear to be safe could have the potent capacity to activate both innate and adaptive immunity. DC stimulation skews the immune response towards the release of raised levels of IL-12 that is naturally produced by these cells and other immune cells. This secretion can be measured by the production of the active heterodimer referred to as p70. DCs secrete IL-12p70 in response to inflammatory cytokines, bacterial/viral components, and CD40 ligation [56]. DCs stimulated with glycophospholipids reveals the **P4** stimulatory activity (Fig. 6). PIMs and their analogues are known activators of NKT cells [11,57,58] along with by stander cells. Supplementary Fig. 4 shows the activation of naïve spleenocytes with glycophopholipids. Among the test compounds, **P4** has shown higher stimulatory activity in contrast to invariant NKT (iNKT) cells but not total T and B cells. As the novel glycophopholipids employed in the current study mimick the structures of PIMs, we have considered assessing these analogues for their properties to activate $CD4^+$ T cells. The latter is indispensable for the immune system. T cells recognize major histocompatibility complex molecules in complex with peptide, presented by antigen-presenting cells (APCs) and activate the secretion of cytokines [59]. We postulated that activation of $CD4^+$ T cells might play a major role in the significant increase in total T cells population [60]. IFN-γ is critical for innate and adaptive immunity. It is produced predominantly by NK and NKT cells (innate) and $CD4^+$ (Th1) and $CD8^+$ T cells (adaptive). IFN-γ derived from NK cells is essential against infectious diseases but not sufficient without $CD4^+$ T cell activation. Adil *et al.*, reported that IFN-γ derived from the $CD4^+$ T cells are indispensable against leishmania or *Listeria monocytogenes* [61]. Our results on antigen presenting cells (DCs) and APCs co-culture with $CD4^+$ T cells along with **P4** supported the aforementioned results (Figs. 7 & 8).

To decipher the immunomodulatory effects of **P4** further, we evaluated its adjuvant activity *in vivo* against OVA in mice and used QS-21 as a standard adjuvant for comparison (Supplementary Fig. 2). *In vivo* studies with model antigen (OVA) revealed that **P4** significantly increased the antigen-specific antibody titer (IgG), along with subclasses (IgG1 and IgG2a) (Fig. 10) and splenocyte proliferation (LPS mediated) (Fig. 11), $CD4^+$ T cell stimulation (Fig. 12), and both Th1 and Th2 cytokine secretion (Fig. 13) in comparison to antigen alone treated groups. However, adjuvant activity of **P4** was not superior to standard QS-21.

In conclusion, novel glycophospholipids were synthesized and evaluated for their safety and immunological characteristics. The molecular docking studies revealed moderate to high docking score indicating the target specificity on DC-SIGN receptors of the host, which provides a clue on their suitability as efficient cellular immune response modulators against virulent pathogens. The glycophospholipids showed no toxicity on murine immune cells (macrophages, DCs, and $CD4^+$ T cells) and human PBMCs at indicated concentrations. Analogues strongly induced danger signals on macrophages through the release of NO, IL-6, and IL-12 and also showed endocytosis of model antigen. IL-12 release from DCs indicated the direct activation signals of glycophospholipids. *In vitro* studies on the heterogeneous population (spleen cells) showed activation of immune cells (T, B, NK, and NKT) upon compound treatment. *In vivo* adjuvant activity of **P4** with model antigen (OVA) although less efficient compared to QS-21, has shown significant efficacy compared, in all assays, with the OVA-alone treated group.

Since it is well established that the dose and efficacy of adjuvants may change based on the type of antigen and type of immune response that is required, **P4** undoubtedly represents a promising adjuvant candidate for further investigation. Finally, in-silico studies strengthen the obtained results from *in vitro* and *in vivo* studies and lead to the conclusion that DC-SIGN may be most likely target involved in its mode of action. This study potentiated the PIMs as immunomodulators, which ultimately needs to be focused in the future to tailor-made the parent PIMs.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Abbreviations

CD	cluster of differentiation
DAMP	damage-associated molecular pattern
DME	dimethoxyethane
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
DMSO	dimethylsulfoxide
SAR	structure – activity relationship
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IFN-γ	interferon gamma
IL	interleukin

LPS	lipopolysaccharide
OVA	ovalbumin
TNF-α	tumor necrosis factor alpha
QS	*Quillaja saponaria*
PBMC	peripheral blood mononuclear cell
Pan-DC	plasmacytoid DC
TMB	3,3′,5,5′-Tetramethylbenzidine
MAb	Monoclonal Antibody
FITC	fluorescein isothiocyanate
APC Cy7	Allophycocyanin Cy7
PE	Phycoerythrin
TCR	T-cell receptor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

Appendix A. Supplementary data

The Supporting Information is available on the website. Experimental data and NMR spectra of synthesized compounds and copies of NMR spectra on the probes; immunological studies protocols.

References

[1] C. Fenselau, E. Gelpi, Mass Spectrometry for the Characterization of Microorganisms, ACS Publications, 1994.
[2] E. Barreto-Bergter, R.T. Figueiredo, Fungal glycans and the innate immune recognition, Front. Cell. Infect. Microbiol. 4 (2014) 145.
[3] C.H. Liu, H. Liu, B. Ge, Innate immunity in tuberculosis: host defense vs pathogen evasion, Cell. Mol. Immunol. 14 (12) (2017) 963–975.
[4] M. Denis, G.D. Ainge, D.S. Larsen, W.B. Severn, G.F. Painter, A synthetic analogue of phosphatidylinositol mannoside is an efficient adjuvant, Immunopharmacol. Immunotoxicol. 31 (4) (2009) 577–582.
[5] G.D. Sprott, C.J. Dicaire, K. Gurnani, S. Sad, L. Krishnan, Activation of dendritic cells by liposomes prepared from phosphatidylinositol mannosides from Mycobacterium bovis bacillus Calmette-Guerin and adjuvant activity in vivo, Infect. Immun. 72 (9) (2004) 5235–5246.
[6] S.R. Bonam, C.D. Partidos, S.K.M. Halmuthur, S. Muller, An overview of novel adjuvants designed for improving vaccine efficacy, Trends Pharmacol. Sci. 38 (9) (2017) 771–793.
[7] P.J. Brennan, Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis, Tuberculosis 83 (1–3) (2003) 91–97.
[8] E. Ishikawa, D. Mori, S. Yamasaki, Recognition of mycobacterial lipids by immune receptors, Trends Immunol. 38 (1) (2017) 66–76.
[9] D. Dao, L. Kremer, Y. Guérardel, A. Molano, W. Jacobs, S. Porcelli, V. Briken, Mycobacterium tuberculosis lipomannan induces apoptosis and interleukin-12 production in macrophages, Infect. Immun. 72 (4) (2004) 2067–2074.
[10] G.D. Ainge, W.J. Martin, B.J. Compton, C.M. Hayman, D.S. Larsen, S.I. Yoon, I.A. Wilson, J.L. Harper, G.F. Painter, Synthesis and Toll-like receptor 4 (TLR4) activity of phosphatidylinositol dimannoside analogues, J. Med. Chem. 54 (20) (2011) 7268–7279.
[11] Y. Kinjo, E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M.R. Benhnia, D.M. Zajonc, G. Ben-Menachem, G.D. Ainge, G.F. Painter, et al., Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria, Nat. Immunol. 7 (9) (2006) 978–986.
[12] B.S. Dyer, J.D. Jones, G.D. Ainge, M. Denis, D.S. Larsen, G.F. Painter, Synthesis and structure of phosphatidylinositol dimannoside, J. Org. Chem. 72 (9) (2007) 3282–3288.
[13] S. Brument, C. Cheneau, Y. Brissonnet, D. Deniaud, F. Halary, S. Gouin, Polymeric mannosides prevent DC-SIGN-mediated cell-infection by cytomegalovirus, Org. Biomol. Chem. 15 (36) (2017) 7660–7671.
[14] N.N. Driessen, R. Ummels, J.J. Maaskant, S.S. Gurcha, G.S. Besra, G.D. Ainge, D.S. Larsen, G.F. Painter, C.M. Vandenbroucke-Grauls, J. Geurtsen, Role of phosphatidylinositol mannosides in the interaction between mycobacteria and DC-SIGN, Infect. Immun. 77 (10) (2009) 4538–4547.
[15] L.C.J. Abel, L.R.P. Ferreira, I. Cunha Navarro, M.A. Baron, J. Kalil, R.T. Gazzinelli, L.V. Rizzo, E. Cunha-Neto, Induction of IL-12 production in human peripheral monocytes by Trypanosoma cruzi is mediated by glycosylphosphatidylinositol-anchored mucin-like glycoproteins and potentiated by IFN-γ and CD40-CD40L interactions, Mediat. Inflamm. 2014 (2014).
[16] L. Proudfoot, A.V. Nikolaev, G.-J. Feng, W. Wei, M. Ferguson, J.S. Brimacombe, F.Y. Liew, Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of Leishmania lipophosphoglycan in murine macrophages, Proc. Natl. Acad. Sci. U. S. A. 93 (20) (1996) 10984–10989.
[17] G.E. Kaiko, J.C. Horvat, K.W. Beagley, P.M. Hansbro, Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123 (3) (2008) 326–338.
[18] G.D. Ainge, N.A. Parlane, M. Denis, C.M. Hayman, D.S. Larsen, G.F. Painter, Phosphatidylinositol mannosides: synthesis and adjuvant properties of phosphatidylinositol di-and tetramannosides, Biorg. Med. Chem. 14 (22) (2006) 7615–7624.
[19] X.Y. Meng, H.X. Zhang, M. Mezei, M. Cui, Molecular docking: a powerful approach for structure-based drug discovery, Curr. Comput. Aided Drug Des. 7 (2) (2011) 146–157.
[20] L. Barbieri, V. Costantino, E. Fattorusso, A. Mangoni, N. Basilico, M. Mondani, D. Taramelli, Immunomodulatory α-Galactoglycosphingolipids: synthesis of 2′-Fluoro-2′-deoxy-α-galactosylceramide and an evaluation of its Immunostimulating properties, Eur. J. Org. Chem. 2005 (15) (2005) 3279–3285.
[21] A. Sandeep, B.S. Reddy, I. Hyder, H.M.S. Kumar, Synthesis of a new class of glycolipids and the evaluation of their immunogenicity using murine splenocytes, J. Carbohydr. Chem. 35 (6) (2016) 326–343.
[22] A.M. Pierdomenico, A. Recchiuti, F. Simiele, M. Codagnone, V.C. Mari, G. Davì, M. Romano, MicroRNA-181b regulates ALX/FPR2 receptor expression and pro-resolution signaling in human macrophages, J. Biol. Chem. 290 (6) (2015) 3592–3600.
[23] T. Sidiq, A. Khajuria, P. Suden, S. Singh, N. Satti, K. Suri, V. Srinivas, E. Krishna, R. Johri, A novel sarsasapogenin glycoside from Asparagus racemosus elicits protective immune responses against HBsAg, Immunol. Lett. 135 (1–2) (2011) 129–135.
[24] N. Takeno, A. Inujima, K. Shinohara, M. Yamada, N. Shibahara, H. Sakurai, I. Saiki, K. Koizumi, Immune adjuvant effect of Juzentaihoto, a Japanese traditional herbal medicine, on tumor vaccine therapy in a mouse model, Int. J. Oncol. 47 (6) (2015) 2115–2122.
[25] C.T. Ng, M.B. Oldstone, Infected CD8α− dendritic cells are the predominant source of IL-10 during establishment of persistent viral infection, Proc. Natl. Acad. Sci. U. S. A. 109 (35) (2012) 14116–14121.
[26] R.N. Mahon, R.E. Rojas, S.A. Fulton, J.L. Franko, C.V. Harding, W.H. Boom, Mycobacterium tuberculosis cell wall glycolipids directly inhibit CD4+ T-cell activation by interfering with proximal T-cell-receptor signaling, Infect. Immun. 77 (10) (2009) 4574–4583.
[27] S. Blüml, G. Zupkovitz, S. Kirchberger, M. Seyerl, V.N. Bochkov, K. Stuhlmeier, O. Majdic, G.J. Zlabinger, C. Seiser, J. Stöckl, Epigenetic regulation of dendritic cell differentiation and function by oxidized phospholipids, Blood 114 (27) (2009) 5481–5489.
[28] C. Saha, M. Das, E. Stephen-Victor, A. Friboulet, J. Bayry, S.V. Kaveri, Differential effects of Viscum album preparations on the maturation and activation of human dendritic cells and CD4+ T cell responses, Molecules 21 (7) (2016) 912.
[29] A.I. Colovai, C. Giatzikis, E.K. Ho, M. Farooqi, N. Suciu-Foca, G. Cattoretti, A. Orazi, Flow cytometric analysis of normal and reactive spleen, Mod. Pathol. 17 (8) (2004) 918–927.
[30] N. Nalla, P. Pallavi, B.S. Reddy, S. Miryala, V.N. Kumar, M. Mahboob, M.S.K. Halmuthur, Design, synthesis and immunological evaluation of 1, 2, 3-triazole-tethered carbohydrate–Pam3Cys conjugates as TLR2 agonists, Biorg. Med. Chem. 23 (17) (2015) 5846–5855.
[31] J. Arnold, D. Murera, F. Arbogast, J.D. Fauny, S. Muller, F. Gros, Autophagy is dispensable for B-cell development but essential for humoral autoimmune responses, Cell Death Differ. 23 (5) (2016) 853–864.
[32] D. Bhunia, P.M. Pallavi, S.R. Bonam, S.A. Reddy, Y. Verma, M.S.K. Halmuthur, Design, synthesis, and evaluation of novel 1, 2, 3-triazole-tethered glycolipids as vaccine adjuvants, Arch. Pharm. 348 (10) (2015) 689–703.
[33] A. Khajuria, A. Gupta, F. Malik, S. Singh, J. Singh, B. Gupta, K. Suri, P. Suden, V. Srinivas, K. Ella, A new vaccine adjuvant (BOS 2000) a potent enhancer mixed Th1/Th2 immune responses in mice immunized with HBsAg, Vaccine 25 (23) (2007) 4586–4594.
[34] P.A. Roche, K. Furuta, The ins and outs of MHC class II-mediated antigen processing and presentation, Nat. Rev. Immunol. 15 (4) (2015) 203–216.
[35] M. Merad, P. Sathe, J. Helft, J. Miller, A. Mortha, The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting, Annu. Rev. Immunol. 31 (2013) 563–604.
[36] N. Frison, M. Taylor, E. Soilleux, M. Bousser, R. Mayer, M. Monsigny, K. Drickamer, A. Roche, Oligolysine-based oligosaccharide clusters: selective recognition and endocytosis by the mannose receptor and dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin, J. Biol. Chem. 278 (26) (2003) 23922–23929.
[37] T.B. Geijtenbeek, S.J. Van Vliet, E.A. Koppel, M. Sanchez-Hernandez, C.M. Vandenbroucke-Grauls, B. Appelmelk, Y. Van Kooyk, Mycobacteria target DC-SIGN to suppress dendritic cell function, J. Exp. Med. 197 (1) (2003) 7–17.
[38] M.E. Rodriguez, C.M. Loyd, X. Ding, A.F. Karim, D.J. McDonald, D.H. Canaday, R.E. Rojas, Mycobacterial phosphatidylinositol mannoside 6 (PIM6) up-regulates TCR-triggered HIV-1 replication in CD4+ T cells, PLoS One 8 (11) (2013) e80938.
[39] S.I. Gringhuis, J. den Dunnen, M. Litjens, B. van het Hof, Y. van Kooyk, T.B. Geijtenbeek, C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB, Immunity 26 (5) (2007) 605–616.
[40] S. Cardinaud, A. Urrutia, A. Rouers, P.G. Coulon, J. Kervevan, C. Richetta, A. Bet, E.A. Maze, M. Larsen, M.C. Iglesias, Triggering of TLR-3,-4, NOD2, and DC-SIGN reduces viral replication and increases T-cell activation capacity of HIV-infected human dendritic cells, Eur. J. Immunol. 47 (5) (2017) 818–829.
[41] F. Debierre-Grockiego, M.A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M.G. Resende, D.S. Mansur, R. Weingart, R.R. Schmidt, D.T. Golenbock, Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii, J. Immunol. 179 (2) (2007) 1129–1137.
[42] G.D. Ainge, N.A. Parlane, M. Denis, B.S. Dyer, A. Härer, C.M. Hayman, D.S. Larsen, G.F. Painter, Phosphatidylinositol mannoside ether analogues: syntheses and interleukin-12-inducing properties, J. Org. Chem. 72 (14) (2007) 5291–5296.
[43] H. Brightbill, R. Modlin, Toll-like receptors: molecular mechanisms of the mammalian immune response, Immunology 101 (1) (2000) 1–10.

[44] B. Billack, Macrophage activation: role of toll-like receptors, nitric oxide, and nuclear factor kappa B, Am. J. Pharm. Educ. 70 (5) (2006) 102.
[45] A. Aderem, D.M. Underhill, Mechanisms of phagocytosis in macrophages, Annu. Rev. Immunol. 17 (1) (1999) 593–623.
[46] A. Berzi, S. Ordanini, B. Joosten, D. Trabattoni, A. Cambi, A. Bernardi, M. Clerici, Pseudo-mannosylated DC-SIGN ligands as immunomodulants, Sci. Rep. 6 (2016) 35373.
[47] A. Berzi, N. Varga, S. Sattin, P. Antonazzo, M. Biasin, I. Cetin, D. Trabattoni, A. Bernardi, M. Clerici, Pseudo-mannosylated DC-SIGN ligands as potential adjuvants for HIV vaccines, Viruses 6 (2) (2014) 391–403.
[48] G.D. Arango, M. Fukuda, S. Turco, S. Stäger, A. Descoteaux, Leishmania promastigotes induce cytokine secretion in macrophages through the degradation of synaptotagmin XI, J. Immunol. 193 (5) (2014) 2363–2372.
[49] J. Mercer, U.F. Greber, Virus interactions with endocytic pathways in macrophages and dendritic cells, Trends Microbiol. 21 (8) (2013) 380–388.
[50] T.H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses, Clin. Microbiol. Rev. 22 (2) (2009) 240–273.
[51] G. Kindberg, S. Magnusson, T. Berg, B. Smedsrød, Receptor-mediated endocytosis of ovalbumin by two carbohydrate-specific receptors in rat liver cells. The intracellular transport of ovalbumin to lysosomes is faster in liver endothelial cells than in parenchymal cells, Biochem. J. 270 (1) (1990) 197–203.
[52] S.E. Autenrieth, I.B. Autenrieth, Variable antigen uptake due to different expression of the macrophage mannose receptor by dendritic cells in various inbred mouse strains, Immunology 127 (4) (2009) 523–529.
[53] E. Doz, S. Rose, V. Vasseur, S. Charron, M. Gilleron, G. Puzo, I. Fremaux, Y. Delneste, F. Erard, B. Ryffel, Mycobacterial phosphatidylinositol mannosides negatively regulate host toll-like receptor 4, MyD88-dependent proinflammatory cytokines, and TRIF-dependent Co-stimulatory molecule expression, J. Biol. Chem. 284 (35) (2009) 23187–23196.
[54] B. Lepenies, J. Lee, S. Sonkaria, Targeting C-type lectin receptors with multivalent carbohydrate ligands, Adv. Drug Del. Rev. 65 (9) (2013) 1271–1281.
[55] B.P. Mahon, M.S. Ryan, F. Griffin, K. Mills, Interleukin-12 is produced by macrophages in response to live or killed Bordetella pertussis and enhances the efficacy of an acellular pertussis vaccine by promoting induction of Th1 cells, Infect. Immun. 64 (12) (1996) 5295–5301.
[56] X. Ma, W. Yan, H. Zheng, Q. Du, L. Zhang, Y. Ban, N. Li, F. Wei, Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells, F1000Research 4 (2015).
[57] K. Fischer, E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S.H. Kaufmann, Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells, Proc. Natl. Acad. Sci. U. S. A. 101 (29) (2004) 10685–10690.
[58] Y.K. Verma, B.S. Reddy, M.S. Pawar, D. Bhunia, H.M. Sampath Kumar, Design, synthesis, and immunological evaluation of benzyloxyalkyl-substituted 1, 2, 3-triazolyl α-GalCer analogues, ACS Med. Chem. Lett. 7 (2) (2015) 172–176.
[59] A.M. Green, R. DiFazio, J.L. Flynn, IFN-γ from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection, J. Immunol. 190 (1) (2013) 270–277.
[60] M.F. Bachmann, A. Oxenius, Interleukin 2: from immunostimulation to immunoregulation and back again, EMBO Rep. 8 (12) (2007) 1142–1148.
[61] A.E. Wakil, Z.E. Wang, J.C. Ryan, D.J. Fowell, R.M. Locksley, Interferon γ derived from CD4+ T cells is sufficient to mediate T helper cell type 1 development, J. Exp. Med. 188 (9) (1998) 1651–1656.