



Immunomodulatory nanoparticles as adjuvants and allergen-delivery system to human dendritic cells: Implications for specific immunotherapy

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ABSTRACT

Novel adjuvants and antigen-delivery systems with immunomodulatory properties that shift the allergic Th2 response towards a Th1 or regulatory T cell response are desired for allergen-specific immunotherapy. This study demonstrates that 200-nm sized biodegradable poly(γ -glutamic acid) (γ -PGA) nanoparticles (NPs) are activators of human monocyte-derived dendritic cells (MoDCs). γ -PGA NPs are efficiently internalized by immature MoDCs and strongly stimulate production of chemokines and inflammatory cytokines as well as up-regulation of co-stimulatory molecules and immunomodulatory mediators involved in efficient T cell priming. Furthermore, MoDCs from allergic subjects stimulated *in vitro* with a mixture of γ -PGA NPs and extract of grass pollen allergen *Phleum pratense* (*Phl p*) augment allergen-specific IL-10 production and proliferation of autologous CD4⁺ memory T cells. Thus, γ -PGA NPs are promising as sophisticated adjuvants and allergen-delivery systems in allergen-specific immunotherapy.

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1. Introduction

The prevalence of type I allergy has increased during the last decades. In Europe, timothy grass (*Phleum pratense*) is one of the most common airborne pollens [1] with *Phl p 5* being one of the major allergens [2]. Seasonal allergic rhinitis is characterized by events that are under regulation of T helper 2 (Th2) cells, i.e. CD4⁺ T lymphocytes that produce high levels of IL-4, IL-5 and IL-13, but low amounts of IFN- γ . So far, the only curative treatment for allergy is specific immunotherapy (SIT), where an immunologic hyporesponsiveness is induced by administration of gradually increasing quantities of allergen extract. SIT is accompanied by suppression of Th2 cells and generation of allergen-specific regulatory T cells (Treg), with increased secretion of IL-10 and TGF- β and development of peripheral tolerance [3]. Dendritic cells (DCs) are essential in the regulation of immune responses as they modulate and control the development of different Th and regulatory T cell

subsets depending on their state of differentiation and maturation [4].

Despite the efficacy of SIT, there are concerns with the treatment including difficulties in standardization of allergen extracts, patient compliance due to frequent injections and long treatment duration, and safety issues comprising local side-effects and risk of anaphylaxis. Non-invasive and patient compliant mucosal immunization, which only requires low doses of antigen to induce both mucosal and systemic immune responses, is an attractive approach to improve SIT. Mucosal administration of soluble antigens is generally recognized to lead to induction of an antigen-specific immunological hyporesponsiveness, termed mucosal tolerance, and treatment with allergen peptides containing major T cell epitopes have proved to be highly effective in preventing allergic disease [5–8]. Another strategy to improve vaccines for SIT is to use adjuvants that *per se* can modulate the allergen-specific T cell response. For this purpose, particle based antigen-delivery systems have shown promising results [9,10]. For instance, allergen-loaded poly(D,L-lactic-co-glycolic) acid (PGLA)-nanoparticles have been shown to modulate an ongoing Th2 response in a murine model after only a few administrations [10].

Self-assembled polymeric nanoparticles, composed of biodegradable poly(γ -glutamic acid) (γ -PGA) and L-phenylalanine ethylester, have been extensively investigated in mice for their potential to be used as protein carriers and adjuvants for DC maturation [11–13]. γ -PGA is a high molecular weight polypeptide

Abbreviations: DC, dendritic cell; MAPK, mitogen-activated protein kinase; MoDC, monocyte-derived dendritic cell; NF- κ B, nuclear factor-kappa B; NP, nanoparticle; PmB, polymyxin B; PBMC, peripheral blood mononuclear cell; *Phl p*, *Phleum pratense*; γ -PGA, poly(γ -glutamic acid); SIT, specific immunotherapy; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell.

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composed of γ -linked glutamic acid units and α -carboxylate side chains produced by certain strains of *Bacillus* [14]. The amphiphilic nature of the hydrophobically modified γ -PGA copolymer enables it to spontaneously form nanoparticles with the ability to encapsulate or immobilize a variety of molecules [15]. Spherical γ -PGA nanoparticles with 53% of phenylalanine grafting in the size range of 100–200 nm have been reported to induce both cellular and humoral antigen-specific immune responses in a murine model [11].

In this study, we investigated the immunoregulatory properties of 200-nm sized γ -PGA NPs, for the purpose of developing a novel adjuvant and allergen-delivery system for SIT, with potential nasal administration. We demonstrate that γ -PGA NPs are rapidly internalized by human monocyte-derived dendritic cells (MoDCs), a process associated with drastic morphological changes. NPs also induce DC maturation with up-regulation of co-stimulatory molecules and production of immunomodulatory cytokines and chemokines. Genome-wide transcriptional analysis of MoDCs and nasal mononuclear cells reveals that NPs induce production of inflammatory mediators associated with Th1 responses and that the stimulatory effect of NPs is largely directed to DCs, indicating that γ -PGA NPs can be used to specifically target DCs. Furthermore, we show that NPs activate DCs through interactions with Toll-like receptor (TLR) 2 and 4. Finally, we demonstrate that NPs significantly augment allergen-specific T cell proliferation, as well as IL-10 cytokine production. In contrast, IL-4, IL-5, IL-13 or IFN- γ cytokine production was unaltered.

In conclusion, our results suggest the possibility that γ -PGA NPs are potent adjuvants for SIT due to their strong capacity to activate DCs and to their ability to expand allergen-specific IL-10-producing memory T cells.

2. Materials and methods

2.1. Reagents

Cell culture medium RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 2 mM L-glutamine and 50 μ g/ml gentamicin (Sigma–Aldrich, St. Louis, MO, USA) was supplemented with either 5 or 10% (v/v) heat inactivated fetal bovine serum (Invitrogen) and is referred to as R5 and R10 medium, respectively. Recombinant human (rh) GM-CSF was purchased from Bayer Healthcare Pharmaceuticals (Montville, NJ, USA) and rhIL-4 from R&D Systems (Minneapolis, MN, USA). The *Phleum pratense* extract (*Phl p*) was a generous gift from Dr. P.A. Würtzen (ALK-Abello, Hørsholm, Denmark, endotoxin level: <0.0025 EU/ml culture) and recombinant *Phl p* 5 was purchased from Biomay (Vienna, Austria). Lyophilized γ -PGA nanoparticles with 53% of phenylalanine grafting (γ -PGA NP) with the size of 200 nm (referred to as NP), were provided by Prof. M. Akashi (Osaka University, Osaka, Japan) and the synthetic procedures have been described elsewhere [14]. The endotoxin content of the γ -PGA NPs used in this study was 0.06 EU/mg of protein, as determined by Limulus Amebocyte Lysate (LAL) assay (Endospecy ES-24S kit, Seikagaku Biobusiness Corp., Japan).

2.2. Flow cytometric analysis

Flow cytometry analysis was performed using FACScan and FACSAria (BD Biosciences, San José, CA, USA). To avoid unspecific Ab binding, cells were incubated with Chrome Pure Mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) prior to staining with fluorescently labelled Abs for 15 min at 4 °C. PBS containing 0.5% BSA (Saveen Werner, Limhamn, Sweden) and 2 mM EDTA (Merck, Whitehouse, NJ, USA) was used in all cell-labelling and washing steps. The following fluorescently labelled

Abs were used in flow cytometry: CD1a-FITC, CD14-PE, CD86-FITC, CD3-PerCy5 (Dako Cytomation, Glostrup, Denmark), CD40-FITC, CD80-PE, HLA-DR-PE, CD3-FITC, CD4-FITC, CD45RO-PE, CD19-PB, CD11c-APC, CD123-APCCy7, IL-4-PE, IL-13-PE (BD Biosciences), IL-10-FITC (Invitrogen), IL-5-FITC (R&D Systems), IL-17-PerCpCy5.5 and IFN- γ -PerCpCy5.5 (eBioscience, San Diego, CA, USA). Unconjugated anti-CD8, CD45RA and glycophorin A Abs were purchased from Dako Cytomation. 7-AAD and Annexin V-FITC (BD Biosciences) were used to assess cell viability. 10,000 events were acquired and gates were set to include only viable cells of interest, based on forward and side scatter characteristics. CellQuest and FACSDiva software (BD Biosciences) were used for data acquisition with FACScan, FACSCanto II or FACSAria. Further data analysis was performed using FCS Express V3 (De Novo Software, Los Angeles, CA, USA).

2.3. Human blood and tissue samples

Peripheral blood mononuclear cells (PBMC) from healthy and allergic individuals were isolated from peripheral blood (Lund University Hospital, Lund, Sweden). The 11 allergic donors in this study tested positive for *Phleum pratense* (*Phl p*) in skin prick test (Soluprick SQ, ALK-Abelló) and had a history of intermittent seasonal allergic rhinitis (Supplementary Table 1). The allergic donors were subjected to an artificial pollen season as previously described [16,17]. Briefly, allergen challenges were given once daily for 7 days after a first blood withdrawal, and at that point, all donors had symptoms of ongoing allergic rhinitis. The study was approved by the regional Ethics Committee and informed consent was obtained. Nasal biopsies were obtained from healthy individuals subjected to nasal obstruction surgery (septoplasty and mucosal reduction). Single cell suspensions were prepared by enzymatic digestions at 37 °C by collagenase (2 mg/ml) and DNase (≥ 0.25 mg total protein) (Sigma–Aldrich) for 20 min. Cells were filtered through a 70 μ m cell strainer (BD Biosciences) and mononuclear cells were isolated by Ficoll Paque centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden). The cells were stained with CD19-PB, CD3-FITC, HLA-DR-PE and CD11c-APC (BD Biosciences) and analyzed using FACSAria. The isolated cell samples contained 23–34% CD3⁺, 2–4% CD19⁺ and 4–9% CD11c⁺/HLA-DR⁺ cells.

2.4. Internalization of γ -PGA NPs

In order to generate immature MoDCs, monocytes were purified from peripheral blood and cultured for 7 days in R10 medium supplemented with rhIL-4 (50 ng/ml) and rhGM-CSF (150 ng/ml) as described elsewhere [18]. Immature MoDCs (2.5×10^5 cells) were incubated for 30 min with 100 μ g/ml FITC-conjugated γ -PGA NPs at 0 and 37 °C, and analyzed using FACScan. Internalization of NPs was further studied with confocal laser scanning microscopy. Alexa Fluor 488-conjugated γ -PGA NPs (100 μ g/ml) were incubated with MoDCs for 30 min and untreated cells were used as control. The cells were washed and immobilized onto glass microscope slides by cytospin centrifugation, counterstained with APC-conjugated anti-HLA-DR (BD Biosciences) and mounted using Prolong Gold Antifade reagent (Invitrogen). The slides were analyzed using laser scanning confocal microscopy (Zeiss LSM 510, Carl Zeiss Jena GmbH, Jena, Germany) at 100 $\times$ magnification. The z-series images obtained were created with LSM 510 software (Carl Zeiss) and ImageJ (<http://rsb.info.nih.gov/ij/>). MoDC morphology was studied during internalization of NPs with time-lapse holographic microscopy using the Holomonitor™ M2 digital holographic microscope/cell analyzer (Phase Holographic Imaging, Lund, Sweden). Immature MoDCs were treated with 100 μ g/ml γ -PGA NPs in cold R10 medium and 30 μ l cell suspensions were applied into the channel of a μ -Slide VI (Ibidi GmbH, München, Germany). The slide was placed

on the microscope connected to a heater keeping the cells at 37 °C. During 30 min, time-lapse sequence images were captured every 10 s with an imaging time of 2.4 ms. The holographic cell analysis was performed using the HoloStudio™ software from Phase Holographic Imaging. Alterations in morphology of one representative cell are shown.

2.5. Phenotypic analysis of MoDCs and assessment of γ -PGA NP cytotoxicity

Immature MoDCs were stimulated for 24 h with 0, 10, 50, 100 and 250 μ g/ml γ -PGA NPs or 0.001–1000 ng/ml LPS (*E. coli* O26:B6; 1,000,000 EU/mg, Sigma–Aldrich) and cell surface expression levels of CD1a, CD14, CD80 and CD86 were analyzed using FACSCanto II. CD86 expression was also assayed on MoDCs stimulated with different concentrations of LPS (0.001–10 ng/ml) $\pm$ γ -PGA NPs (100 μ g/ml), and with LPS (1–100 ng/ml) or γ -PGA NPs (100 μ g/ml) pre-incubated for 1 h in rt $\pm$ 10 μ g/ml polymyxin B (PmB, 8030 units/mg, Sigma–Aldrich). Furthermore, cells were stained with Annexin V and 7-AAD after 48 h for detection of early apoptotic versus late apoptotic or dead cells.

2.6. Microarray sample preparation and data analysis

Immature MoDCs were stimulated for 8 and 24 h with 100 μ g/ml γ -PGA NPs. Mononuclear cells from nasal biopsies were cultured for 8 h with 100 μ g/ml NPs. Unstimulated samples were used as controls. Finally, cells were washed, dissolved in TRIzol Reagent (Invitrogen) and stored at –20 °C until further RNA extraction and analysis. 2–4 μ g total RNA from MoDCs and 30–80 ng total RNA from nasal cells were used to generate cDNA, that was further amplified and converted to labelled cRNA prior to expression analysis, using kits and protocols supplied by the manufacturer (Affymetrix, Santa Clara, CA, USA). Hybridization, washing and scanning of the Human Gene 1.0 ST Arrays (MoDCs) or the Human Genome U133 Plus 2.0 Arrays (nasal cells) were performed according to the manufacturer's protocol (Affymetrix). For data analysis, .cel files were uploaded to GeneSpring GX 10 software (Agilent Technologies, Santa Clara, CA) and normalized using robust multi array (RMA). All data were logarithmic transformed and baseline was set to the median of control (untreated) samples. Data sets were subsequently filtered for genes with signal intensity values above a 20-percentile cut-off. For each donor, genes were selected that displayed an alteration in expression level of at least 1.5-fold after 8 or 24 h of NP-stimulation, compared to untreated cells, as well as a statistical difference in expression levels determined by paired *t*-test, $p < 0.05$. Differentially expressed genes were further analyzed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) to enable identification of functionally related and interacting transcripts in pathways and networks. IPA organizes genes into networks based on relationships and interactions extracted from peer-reviewed scientific literature. Each generated network consists of maximum 35 focus genes and is assigned a *p*-value based significance score (score $\times$ corresponds to a *p*-value of 10^{-X}), indicating the likelihood that the focus genes are found within the network by random chance. Additional information is available at the IPA website: www.ingenuity.com. Functionally associated, differentially expressed, genes were grouped according to their gene ontology (www.affymetrix.com/analysis/index.affx). Genes encoding chemokines, cytokines, growth factors and receptors, adhesion molecules, CD-markers, C-type lectins and molecules associated with the NF- κ B and MAPK pathways or other immune-related functions were selected for visualization.

2.7. Multiplex cytokine and chemokine analysis of MoDC supernatants

Multianalyte profiling of supernatants from MoDCs left untreated or stimulated with 100 μ g/ml γ -PGA NPs for 12 h was performed using Luminex Human Cytokine 25-Plex AB bead kit (Invitrogen), according to the manufacturer's instructions on the Luminex-100 system (Applied Cytometry Systems, Sheffield, UK). Acquired fluorescence data were analyzed using Star Station software (Applied Cytometry Systems). Cytokines assayed were: IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , GM-CSF, MIP-1 α , MIP-1 β , IP-10, MIG, Eotaxin, RANTES and MCP-1.

2.8. TLR blocking

Immature MoDCs were incubated for 1 h with 20 μ g/ml TLR2 or TLR4 neutralizing antibodies (R&D Systems) prior to stimulation with 100 μ g/ml γ -PGA NPs. Untreated cells or cells treated with TLR2 or TLR4 Abs alone were used as controls. Cells were incubated for 48 h and alterations in expression levels of CD1a, CD14, CD40, CD80, CD86 and HLA-DR were analyzed with flow cytometry.

2.9. T cell purification

CD4⁺ memory T cells were negatively selected from PBMCs obtained from allergic subjects after 7 days of allergen challenge, using Dynabeads Untouched Human CD4 T cells kit (Invitrogen) with the addition of CD8, CD45RA and glycophorin A Abs. Cells were incubated for 20 min at 4 °C with the Abs, and thereafter, anti-mouse Depletion MyOne Dynabeads were added for 15 min at RT. Unwanted cells were removed using a Dynal MPC-15 magnet. The cells isolated were >90% pure CD4⁺CD45RO⁺ T cells.

2.10. T cell proliferation induced by γ -PGA NP- and allergen-stimulated MoDCs

MoDCs from allergic individuals ($n = 11$) were stimulated with 25 μ g/ml *Phl p* or 5 μ g/ml *Phl p* 5, alone or in combination with 100 μ g/ml γ -PGA NPs. Untreated or NP-stimulated cells were used as controls. After 48 h, cells were washed twice and subsequently co-cultured with autologous CD4⁺CD45RO⁺ T cells, in a DC:T cell ratio of 1:20, in R5 medium supplemented with 2×10^{-5} M 2-mercaptoethanol (Merck). After 7 days, 2.5 μ Ci/ml [3H]thymidine (Amersham Biosciences, Uppsala, Sweden) was added to each well for 16 h. Thymidine incorporation was measured in a beta scintillation counter (Matrix 96 Direct beta counter, Packard, Meriden, CT). Samples were run in five replicates and the statistical significance was determined by Wilcoxon signed rank test.

2.11. T cell cytokine production induced by γ -PGA NP- and allergen-stimulated MoDCs

CD4⁺CD45RO⁺ T cells from allergic individuals ($n = 11$) were co-cultured with stimulated MoDCs, as described above. For analysis of intracellular cytokine production, cells were incubated with PMA (50 ng/ml), ionomycin (500 ng/ml) and brefeldin A (10 μ g/ml) (Sigma–Aldrich) for 5 h, washed twice in PBS/BSA and fixed with 4% formaldehyde (Pierce Biotechnology, Rockford, IL) for 20 min. Cells were permeabilized with saponin buffer (PBS containing 0.5% saponin (Sigma–Aldrich) and 0.5% BSA) and stained with mAbs specific for IL-4, IL-5, IL-13, IL-10, IL-17 and IFN- γ for 30 min at 4 °C. Thereafter, cells were washed twice in saponin buffer, resuspended in PBS/BSA and analyzed with FACScan.

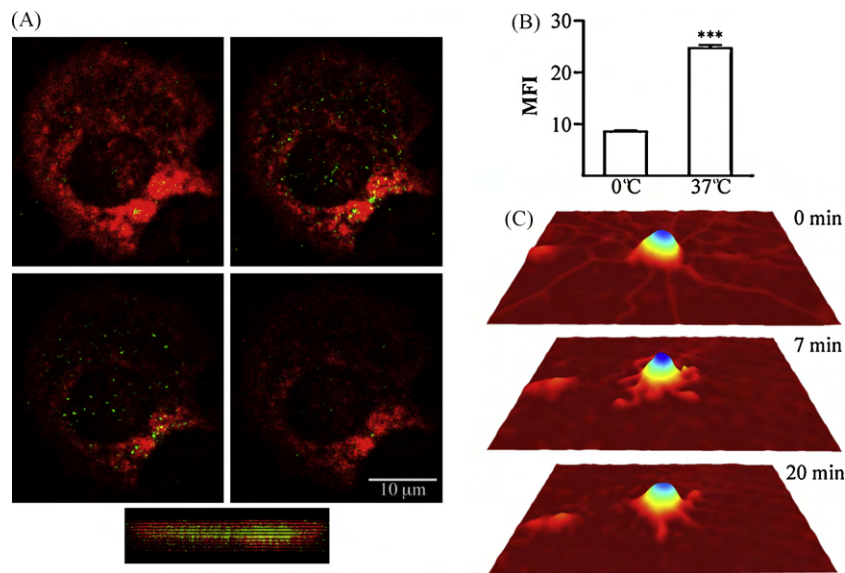


Fig. 1. γ -PGA NPs are efficiently internalized by immature MoDCs, a process associated with drastic morphological alterations. (A) Internalization of Alexa Fluor 488-conjugated γ -PGA NPs (green) after 30 min at 37°C. Cells were immobilized onto glass microscope slides and counterstained with APC-conjugated anti-HLA-DR (red). Confocal laser scanning microscopy images from a z-series of 20 slices are shown. Large panels, x-y sections; small panels, x-z sections. (B) Internalization of FITC-conjugated γ -PGA NPs at 37°C, compared to 0°C. Mean fluorescence intensity (MFI) $\pm$ SEM is represented, $n=9$. The statistical significance was determined by paired Student's two-tailed t -test (*** $p < 0.001$). (C) Holographic microscopy images of one representative MoDC recorded at times 0, 7 and 20 min during internalization of NP-200, starting at the time the cells had sedimented in the chamber and their dendrites were visible.

3. Results

3.1. Internalization of γ -PGA NPs is associated with drastic morphological changes in MoDCs

Internalization of the nanoparticles is a prerequisite to function as antigen-carriers that would enable improved antigen presentation to T cells. Thus, the ability of immature MoDCs to internalize γ -PGA NPs was investigated with flow cytometry and confocal laser scanning microscopy. After 30 min of incubation, Alexa Fluor 488-conjugated γ -PGA NPs were predominantly localized inside the cells (Fig. 1A). Furthermore a 3-fold increase in mean fluorescence intensity (MFI) of cells incubated with FITC-labelled γ -PGA NPs at 37°C compared to the 0°C control (Fig. 1B) was observed. Accordingly, γ -PGA NPs are efficiently internalized by MoDCs. Time-lapse holographic microscopy was used to visualize the effects on DC morphology during internalization of γ -PGA NPs. MoDCs were treated with NPs at 37°C and images were captured every 10 s, during 30 min (Supplementary Video 1). 3D holographic images from the time-lapse sequence (Fig. 1C) visualize the changes in morphology of MoDCs during the course of NP-internalization. Analysis of collected microscopy data demonstrated that the cell area increased with time, whereas cell irregularity and eccentricity, i.e. a measure of how much the shape deviates from a circle,

decreased (data not shown), illustrating the significant change in cellular morphology.

3.2. γ -PGA NPs induce maturation of MoDCs

In order to investigate the effect of γ -PGA NPs on DC maturation, MoDCs were subjected to immunophenotypic analysis by flow cytometry, after 24 h of stimulation. Incubation of MoDCs with different amounts of γ -PGA NPs resulted in up-regulation of CD86 and CD80 in a concentration-dependent manner (Fig. 2). Furthermore, the majority of cells (>95%) treated with 250 μ g/ml of NPs for 48 h were viable, as determined by Annexin V and 7-AAD staining (data not shown).

3.3. γ -PGA NP-mediated MoDC maturation is not caused by contamination with LPS

To exclude the possibility that endotoxin contamination was responsible for the observed maturation, we next assessed the presence of LPS in our preparations. The endotoxin content of 100 μ g/ml γ -PGA NPs was measured to 0.006 EU/ml, which is equivalent to 6 pg/ml of *E. coli* strain O25:B6 LPS. As shown in Fig. 3, up-regulation of CD86 on MoDCs was not induced by LPS-concentrations lower than 10 pg/ml, and thus, NP-mediated MoDC maturation is most

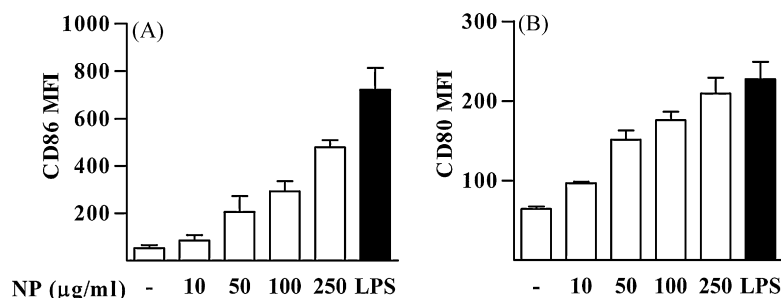


Fig. 2. Stimulation with γ -PGA NPs induces concentration-dependent maturation of MoDCs. Immature MoDCs were stimulated with various concentrations of γ -PGA NPs or 1 μ g/ml LPS for 24 h and surface expression levels of (A) CD86 and (B) CD80 were monitored by flow cytometry. Median fluorescence intensity (MFI) $\pm$ SEM is represented, $n=3$.

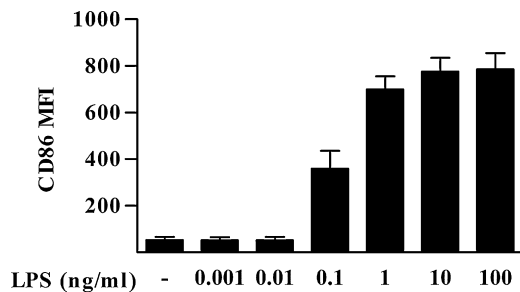


Fig. 3. Stimulation with low concentrations of LPS does not induce MoDC maturation. Immature MoDCs were stimulated with various concentrations of LPS for 24 h and surface expression levels of CD86 were analyzed by flow cytometry. Median fluorescence intensity (MFI) $\pm$ SEM is represented, $n=3$.

likely not caused by contamination with LPS. However, synergistic effects between the NPs and LPS could potentially enable small quantities of LPS to induce maturation. Thus, the synergistic effects of NPs and LPS were investigated by stimulating MoDCs for 24 h with 0.001–10 ng/ml of LPS and/or 100 μ g/ml γ -PGA NPs. As shown in Fig. 4A, no such effect was observed as CD86 expression was not altered when low concentrations of LPS were added together with γ -PGA NPs. Interestingly, γ -PGA NPs instead appeared to have an inhibitory effect on MoDC maturation mediated by high concentrations of LPS. Furthermore, 1, 10 or 100 ng/ml of LPS or 100 μ g/ml γ -PGA NPs were pre-incubated with or without 10 μ g/ml of the LPS-neutralizing agent PmB prior to MoDC stimulation. As shown in Fig. 4B, pre-treatment with PmB completely inhibited LPS-induced up-regulation of CD86 on MoDCs, even at high LPS-concentrations (100 ng/ml). In contrast, the stimulatory capacity of γ -PGA NPs was not significantly affected. Altogether, the results clearly demonstrate that the biological effects generated by γ -PGA NPs are not caused by LPS contamination.

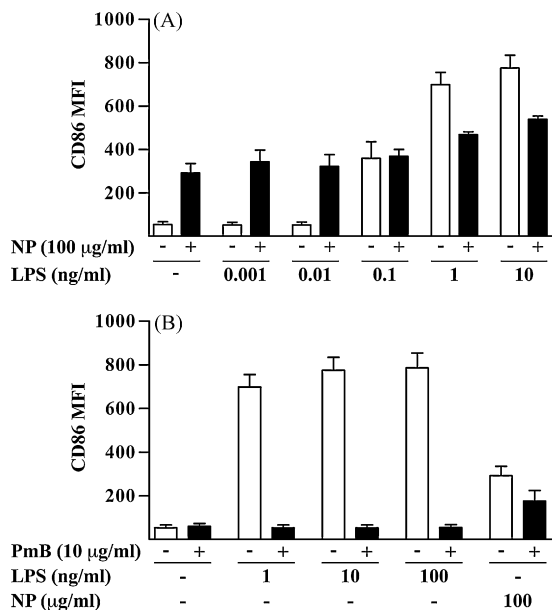


Fig. 4. γ -PGA NP-mediated MoDC maturation is not caused by LPS contamination. (A) Assessment of possible synergistic effects between NPs and LPS on MoDC maturation. Immature MoDCs were left untreated or stimulated with various concentrations of LPS and/or 100 μ g/ml γ -PGA NPs for 24 h. (B) Effect of PmB on LPS- and γ -PGA NP-induced maturation of MoDC. Immature MoDCs were stimulated for 24 h with 0–100 ng/ml of LPS or 100 μ g/ml γ -PGA NPs that had been pre-incubated for 1 h in rt with or without 10 μ g/ml PmB. Surface expression levels of CD86 were analyzed by flow cytometry. Median fluorescence intensity (MFI) $\pm$ SEM is represented, $n=3$.

3.4. Transcriptional activity induced by γ -PGA NPs in MoDCs

Gene expression analysis was performed on MoDCs stimulated with γ -PGA NPs for 8 or 24 h, as compared to untreated cells, in order to investigate the effects of NPs on the transcriptome. Transcripts with at least a 1.5-fold increase or decrease in expression levels after 8 or 24 h in all three donors were selected. Stimulation of MoDCs with NPs resulted in significant ($p < 0.05$) differential expression of 144 unique transcripts (105 up- and 39 down-regulated) after 8 h and 403 transcripts (267 up- and 136 down-regulated) after 24 h. Combining the gene lists regulated after 8 and 24 h of NP-stimulation resulted in a total number of 428 differently expressed transcripts, demonstrating that the majority of regulated genes were similarly expressed at 8 and 24 h (Fig. 5 and Supplementary Table 2). Several genes important for immune function, such as those encoding for CD-markers, cytokines, chemokines and lectins, as well as other immunologically related molecules could be identified (Fig. 5). NP-induced genes were further clustered into networks, according to their interactions, using the IPA software. The IPA analysis of genes differentially regulated after 24 h of NP-stimulation generated five highly significant gene networks. Fig. 6A and B shows the two top-scoring networks, both comprising the maximum number of 35 focus genes and having a high significance score of 52. TNF- α and IL-1 β were found to be central nodes with a high degree of connectivity to other nodes in the networks. The analysis further demonstrated that the biological functions of genes in the dataset were significantly related to cellular growth and proliferation, antigen presentation, cellular movement, cell death and cell-to-cell signalling and interaction.

3.5. mRNA and protein expression profile of chemokines and cytokines induced by γ -PGA NPs

The pronounced up-regulation of a large number of chemokines and cytokines involved in DC migration and activation of immune responses by γ -PGA NPs prompted us to investigate the corresponding protein levels, using multiplex analysis of the culture supernatants. As the mRNA levels of many of the analytes were higher at 8 h compared to 24 h of NP-stimulation, protein analysis was performed on MoDC supernatants 12 h after stimulation. Of the 25 analytes tested by the multiplex assay, 11 cytokines were up-regulated on both mRNA and protein level (Fig. 7A and B). Among them, the chemoattractants IL-8, MIP-1 α (CCL3), MIP-1 β (CCL4) and MCP-1 (CCL2) were highly induced. In addition, a lower induction of the T cell activating chemokines IL-17 and IL-6, as well as the pro-inflammatory cytokines TNF- α , IL-1 β and IL-12 and the soluble receptor IL-2R (CD25) was detected. The IL-12 beta subunit p40 (IL12B) was highly up-regulated on a transcriptional level, whereas the alpha subunit p35 (IL12A) was unaltered. Instead, transcript of the IL-23 alpha subunit p19 (IL23A), which together with IL-12p40 forms the heterodimer IL-23, was up-regulated. Furthermore, moderately high induction of RANTES (CCL5), as well as fairly low induction of IL-15 gene expression was detected; however these two proteins were not identified in MoDC supernatants. Moreover, none of the remaining 13 cytokines included in the multiplex analysis could be detected either on mRNA or protein level.

3.6. Transcriptional activity induced by γ -PGA NPs in nasal mononuclear cells

With the aim of developing a potential nasal vaccination strategy, effect of NPs on nasal tissue cells was evaluated by transcriptional analysis. The nasal biopsy mononuclear cells (4–9% CD11c⁺/HLA-DR⁺/CD19⁻ cells, 2–4% CD19⁺ B cells and 23–43%

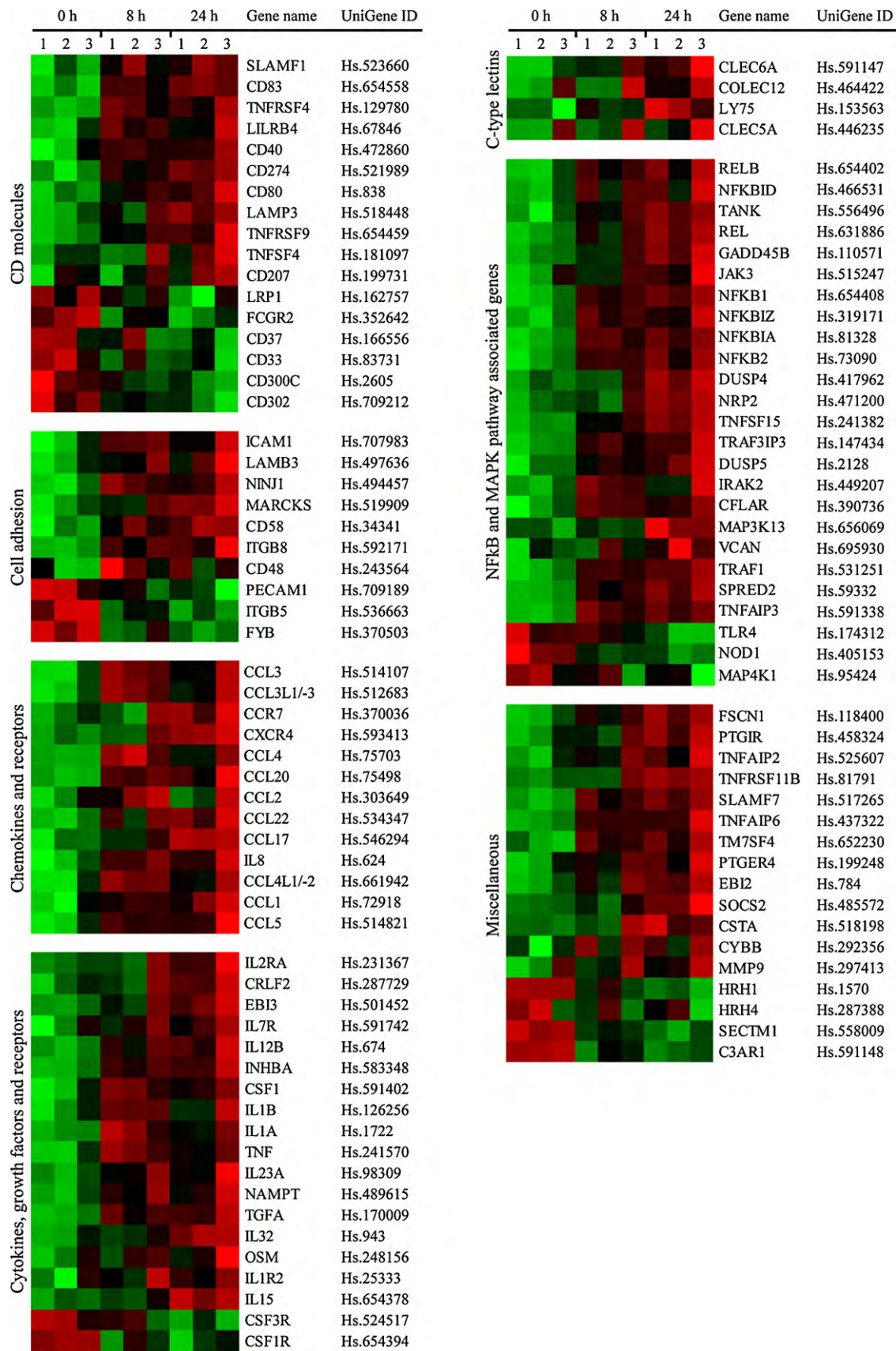


Fig. 5. Stimulation with γ -PGA NPs induces drastic transcriptional activity in DCs. Differentially expressed genes in MoDCs from all donors ($n=3$), filtered on fold change (1.5) and statistical significance ($p < 0.05$) after 8 and/or 24 h of NP-stimulation, as compared to untreated samples, are represented. Adhesion molecules, soluble mediators, receptors, C-type lectins and genes associated with the NF- κ B and MAPK pathways or immune function are visualized with UniGene annotations. The heat map is coloured according to Z-scores of log-transformed intensity values, with red indicating Z-scores > 0 , green indicating Z-scores < 0 and black indicating Z-scores ~ 0 .

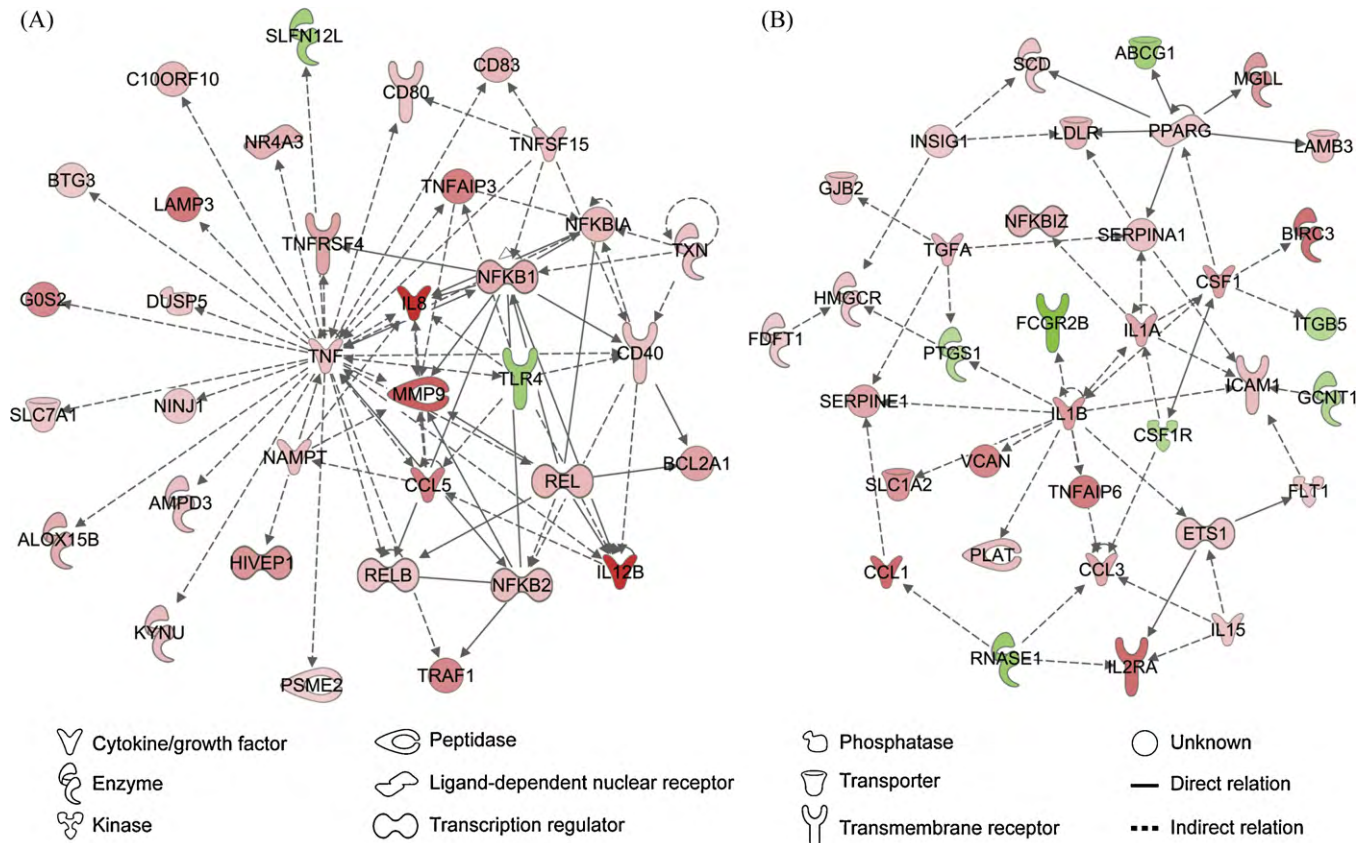


Fig. 6. Stimulation with γ -PGA NPs induces regulation of interconnected genes in MoDCs. Two of the top-scoring networks of genes regulated after 24 h of NP-stimulation, where TNF- α (A) and IL-1 β (B) are central nodes, generated by the Ingenuity Pathway Analysis (IPA) software are shown. Red and green represent up- and down-regulated genes, respectively, based on fold change as compared to untreated samples.

CD3⁺ T cells) were stimulated with NPs for 8 h, which resulted in a limited number of significantly altered genes, compared to untreated cells. Stimulation with NPs resulted in 8 up-regulated and 61 down-regulated transcripts in all of the three donors (Supplementary Table 3). Furthermore, a minor number of transcripts regulated in nasal mononuclear cells were also regulated by NP-stimulation in MoDCs.

3.7. γ -PGA NPs signal through Toll-like receptors

Transcriptional analysis demonstrated an induction of molecules involved in NF- κ B activation by γ -PGA NP-stimulation (Fig. 5), which may reflect TLR/IL-1-receptor mediated activation of DCs. In order to investigate if NPs activate DCs through TLR2 or TLR4, a blocking experiment was performed, using neutralizing

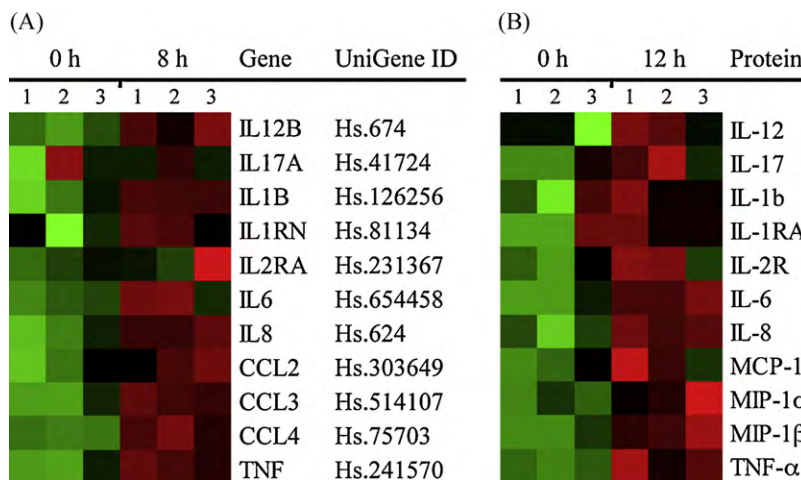


Fig. 7. Stimulation with γ -PGA NPs induces both mRNA expression and secretion of chemokines and interleukins. MoDCs were analyzed with transcriptional microarrays (A) and Luminex multiplex assay (B) after 8 and 12 h of NP-stimulation, respectively. 1–3 denotes data from replicate samples from three healthy donors. Different donors were used for the mRNA and protein assessments. The heat map is coloured according to Z-scores of log-transformed values, with red indicating Z-scores > 0, green indicating Z-scores < 0 and black indicating Z-scores ~ 0.

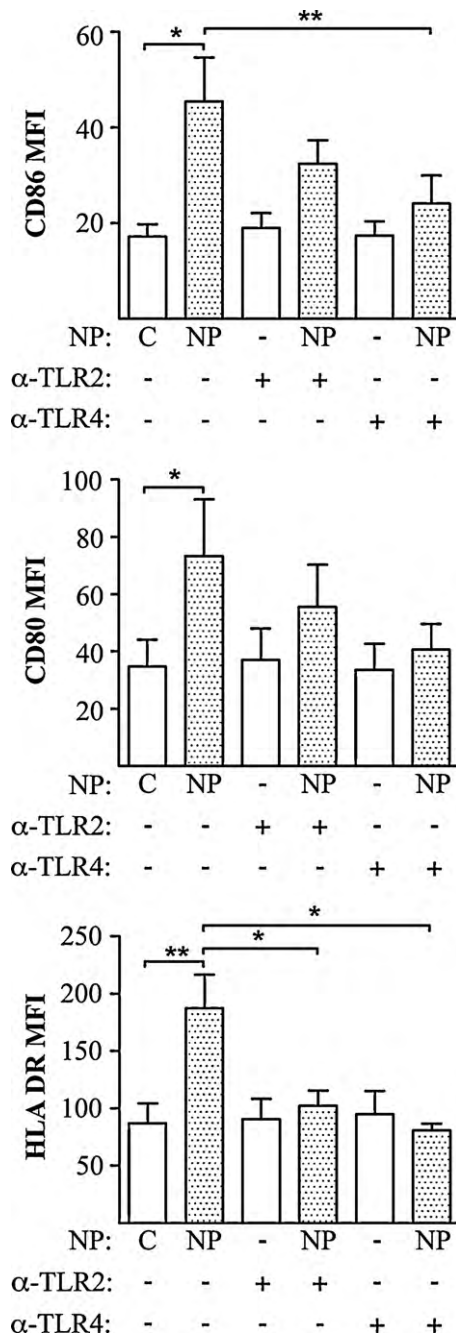


Fig. 8. Blocking of TLR2 or TLR4 on MoDCs decrease the stimulatory effect of γ -PGA NPs. Immature MoDCs were incubated with 20 μ g/ml TLR2 or TLR4 neutralizing Abs for 1 h and thereafter stimulated for 48 h with 100 μ g/ml γ -PGA NPs. Alterations in expression levels of HLA-DR, CD86 and CD80 were analyzed with flow cytometry. C denotes untreated control. Mean fluorescence intensity (MFI) $\pm$ SEM is shown, $n = 5$. The statistical significance was determined by paired Student's two-tailed t -test (* $p < 0.05$, ** $p < 0.01$).

TLR2 and TLR4 antibodies. Stimulation of MoDCs with NPs after pre-incubation with TLR4 antibodies resulted in a significant decrease in CD86 and HLA-DR expression, as well as a marked decrease in CD80 expression, compared to NP-treatment alone (Fig. 8). Blocking of TLR2 also significantly reduced NP-induced HLA-DR expression and a decrease in CD86 and CD80 was also observed (Fig. 8). This data indicates that γ -PGA NPs activate DCs through both TLR2 and TLR4.

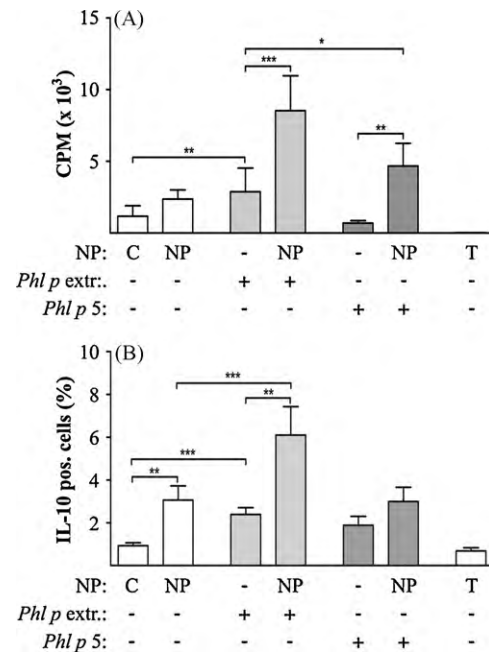


Fig. 9. Stimulation with γ -PGA NPs augments allergen-specific T cell proliferation and IL-10 production. CD4⁺ memory T cells from grass pollen-allergic individuals were co-cultured with autologous MoDCs for 7 days. 48 h prior to co-culture, MoDCs were either left untreated (marked C) or stimulated with 25 μ g/ml *Phl p* extract or 5 μ g/ml recombinant *Phl p* 5, with or without 100 μ g/ml γ -PGA NPs (marked NP). T cells alone were used as control (marked T). Proliferation was assayed by [³H]thymidine incorporation (A). Data represents counts/min (CPM) $\pm$ SEM, $n = 11$ allergic donors. Percentage of IL-10-producing T cells was determined by intracellular flow cytometry (B). Mean values are presented $\pm$ SEM, $n = 11$ allergic donors. The statistical significance was determined by Wilcoxon signed rank test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.8. γ -PGA NPs augment allergen-specific proliferative and cytokine responses by memory CD4⁺ T cells in vitro

In order to evaluate the potential of γ -PGA NPs to function as adjuvants, we next investigated the proliferative and cytokine response induced by allergen. Memory CD4⁺ T cells from grass pollen-allergic subjects were co-cultured with autologous MoDCs, treated with *Phl p* or *Phl p* 5, with or without the presence of NPs. T cell proliferation and intracellular cytokine production were determined, using thymidine incorporation and flow cytometry, respectively. As shown in Fig. 9A and B, extract of *Phl p*, but not recombinant *Phl p* 5, stimulated proliferation of CD4⁺ memory T cells from sensitized subjects and induced IL-10 production. *Phl p* stimulation also significantly increased the frequencies of IL-4 and IL-13 positive T cells, as previously described [19] (data not shown). Administration of *Phl p* extract in combination with NPs significantly augmented both the proliferative T cell response (Fig. 9A), as well as the IL-10 production (Fig. 9B), without affecting the secretion of IL-4 and IL-13 (data not shown). Furthermore, *Phl p* 5 administered with NPs induced T cell proliferation, which was not detected when either NPs or *Phl p* 5 were administered alone. Finally, IFN- γ , IL-5 or IL-17 production was unaltered by stimulation with allergens and/or NPs (data not shown).

4. Discussion

Biodegradable γ -PGA NPs are efficient carriers of multifunctional proteins with potent immunomodulatory activities, as demonstrated by their ability to activate both antigen-specific humoral and cellular immunity by targeting murine DCs [11]. The potential of using γ -PGA NPs in vaccination and for triggering

murine immunity has been demonstrated for several antigens, such as HIV-1 p24 [13], HIV-1 gp120 [12], ovalbumin and peptides of *Listeria monocytogenes* [11]. The present study is the first report describing the stimulatory potential of γ -PGA NPs in humans and we describe the immunoregulatory mechanisms involved in stimulation of human DCs and in allergen-specific T cell activation, for the purpose of developing a novel approach to allergen-specific immunotherapy.

Antigen internalization, presentation and subsequent maturation of DCs, accompanied by phenotypic changes, are essential processes for T cell activation, and thus important factors to consider for the design of novel strategies in immunotherapy. In the present study, we demonstrated that MoDCs efficiently internalized 200-nm sized γ -PGA NPs, a process accompanied by morphological and phenotypical changes including concentration-dependent up-regulation of co-stimulatory CD86 and CD80. Maturation of DCs results in a global transcriptional reprogramming depending on the stimuli [18,20] and, in the present study, it was shown that NPs induced dramatic transcriptional changes in MoDCs with expression profiles similar to those described for DCs matured with inflammatory mediators [18]. Stimulation with γ -PGA NPs induced transcription of a considerable number of chemokines and cytokines, which to a wide extent was confirmed on protein level. Increased mRNA and protein expression of the pro-inflammatory cytokines TNF- α and IL-1 β were identified upon stimulation with NPs, as well as IL-8, MIP-1 α , MIP-1 β and MCP-1, which are important amplifiers of the innate immune response and which recruits monocytes, immature DCs and T cell subsets [21]. Also, secretion of the major Th1-biasing cytokine IL-12 was detected in DC supernatants, as well as increased mRNA levels of IL-12p40, but not p35. In addition, NPs induced transcription of the IL-23 subunit p19. IL-23 has been shown to promote differentiation of Th1 cells in the absence of IL-12, but does in contrast to IL-12 induce proliferation of exclusively memory T cells [22]. Taken together, the administration of γ -PGA NPs appears to be a potent way to skew the environment of polarizing secreted mediators, which may influence the immunological outcome during allergen challenge.

In the present study, gene expression of a considerable number of cell surface markers essential for the ability of DCs to migrate to secondary lymphoid organs and activate naïve T cells were induced by γ -PGA NPs. For instance, a marked up-regulation of CCR7 and CXCR4 enabling co-localization of maturing DC and naïve T cells in secondary lymphoid organs [23,24] was detected as well as up-regulation of adhesion molecules CD48, ICAM-1 (CD54) and CD58 (LFA-3) and matrix metalloproteinase 9 (MMP-9), also involved in cell migration [25]. Furthermore, DC maturation markers CD208 (DC-LAMP) and CD83 were induced by NPs, as well as several co-stimulatory molecules important for T cell activation such as CD40, CD80, CD137 (4-1BB) and the recently discovered CD274 (B7-H1, PD-L1). There are contradicting results concerning CD274, as it has been shown to deliver an inhibitory signal to activated T cells [26] as well as stimulate proliferation and secretion of IL-10 by T cells [27]. CD274 has also been postulated to mediate a negative feedback signal by down-regulating the expression of co-stimulatory molecules and IL-10 production by DCs, thus resulting in a tolerogenic state and the suppression of ongoing immune responses [28]. Moreover, γ -PGA NPs induced up-regulation of immunoglobulin-like transcript 3, ILT3 (LILRB4, CD85k), an inhibitory receptor with a central role in the induction of suppressive T cell responses [29]. Altogether, the present findings show that stimulation with γ -PGA NPs strongly induces surface expression of T cell activating molecules in MoDCs.

Pathway analysis identified various networks of interacting genes expressed upon stimulation with 200-nm sized γ -PGA NPs. The two top-scoring networks recognized TNF- α and IL-1 β as cen-

tral regulatory molecules, with direct or indirect connections to a number of chemokines and interleukins, as well as other molecules relevant for immune function, including CD40, CD80, CD83, DC-LAMP and IL-12p40, involved in antigen presentation and T and/or B cell activation. Furthermore, NPs induced expression of nuclear factor-kappa B (NF- κ B), which is directly triggered upon activation of the TLR/IL-1R family and plays an important role in activation of both innate and adaptive immune responses. Four of the five members of the NF- κ B family; p50 (NF- κ B1), p52 (NF- κ B2), c-REL (REL) and RelB were up-regulated, together with other molecules involved in NF- κ B activation, including VEG1 (TNFSF15) [30], I-TRAF (TANK) [31] and IRAK-2 [32]. Moreover, up-regulation of NF- κ B negative regulators I κ B (NF- κ BIA/NF- κ BID/NF- κ BIZ) [33], A20 (TNFAIP3) [34] and TRAF-1 [35] was detected. Activation of NF- κ B and mitogen-activated protein kinase (MAPK) signalling pathways by γ -PGA NPs has previously been demonstrated in murine DCs [11,36], which is in agreement with the present findings.

With the intention to develop a potential mucosal vaccine adjuvant, we investigated the transcriptional effects induced by γ -PGA NPs on nasal mononuclear cells. *In vitro* stimulation with γ -PGA NPs resulted in modest gene regulation, and the majority of the differentially expressed transcripts were down-regulated. Among the up-regulated immunoregulatory transcripts, we identified CX₃CR1, which is expressed on activated endothelial cells and mediates leukocyte adhesion and migration [37], as well as T cell attracting lymphotactin (XCL-1). Interestingly, lymphotactin has previously been reported to function as a mucosal adjuvant, strongly stimulating Th1 responses with induction of antigen-specific mucosal sIgA and plasma IgG antibodies, but also IgE [38]. Furthermore, lymphotactin has been shown to enhance regulatory activities in CD4⁺CD25^{high}CD127^{low} T cells in allergic asthma [39]. The modest induction of mRNA expression observed in nasal mononuclear cells stimulated by γ -PGA NPs may be explained by the preferential targeting of DCs, which were present at low frequencies in the observed samples. Further studies on individual cell types are necessary to fully determine the specificity of γ -PGA NPs.

In agreement with the present observation involving MoDCs, preliminary data from our laboratory show that γ -PGA NPs induce maturation and transcriptional activity also in human peripheral blood myeloid DCs (mDC), but not plasmacytoid DCs (pDC), suggesting that γ -PGA NPs signal through receptors expressed differently by these DC subsets (Broos et al., unpubl.). This conceptually important finding prompted us to investigate the potential role of TLR2 and TLR4 in NP-signalling, since these particular receptors are expressed by mDCs, but not pDCs [40]. Pre-treatment with both TLR2- and TLR4 neutralizing antibodies clearly inhibited MoDC maturation induced by γ -PGA NPs, implying that these NPs signal through both receptors. Signalling through different TLRs has been shown to conduct distinctive modes of activation [42] and crosstalk between different TLRs may furthermore modify the primary response to their agonist [43]. In agreement with the present finding, γ -PGA has recently been reported to induce activation of murine DCs through TLR4 [41,44]. Furthermore, our data clearly demonstrates that TLR4 activation by γ -PGA NPs is not caused by contamination with LPS. The trace amounts of LPS found in the NP-stimulations were not sufficient to induce MoDC maturation and furthermore, no synergistic effect was observed between the γ -PGA NPs and such small amounts of LPS. In contrast γ -PGA NPs appeared to inhibit LPS-induced MoDC maturation when given together with higher concentrations of LPS. The cause of this effect is currently unknown, but it possible that γ -PGA NPs and LPS compete for the same receptor and thus, LPS-stimulation is partially blocked. In addition, pre-incubation with the LPS-neutralizing agent PmB completely inhibited LPS-induced CD86 expression on MoDCs, whereas the stimulatory capacity of NPs was not significantly affected. This

data is further supported by experiments on murine bone marrow-derived DCs, showing that PmB efficiently inhibits up-regulation of CD86 and CD40, as well as production of TNF- α mediated by LPS, but not by γ -PGA NPs [45]. Altogether, the present results suggest that γ -PGA NPs activates DCs by signalling through both TLR2 and TLR4.

TLR active adjuvants have been extensively investigated in relation to allergen-specific immunotherapy, based on the fundamentals of the hygiene hypothesis suggesting that microbial stimulation of TLRs, which direct the immune system towards Th1 activation, can counterbalance pro-allergic Th2 responses [46]. In this context, TLR2 has specifically been postulated to modulate allergic responses, since increased expression of TLR2 mRNA can be found in children of farmers together with a decreased risk of developing allergies [47]. However, some studies have shown that TLR2 agonists can exacerbate Th2 responses [48,49] while others have shown that they decrease allergen-induced immune responses [50,51]. Moreover, successful suppression of allergic responses has been reported by adjuvants acting either on TLR2 and TLR4, such as the bacterial lipoprotein Opr1 [52], or on TLR4, such as the recently approved adjuvant monophosphoryl lipid A (MPL) [51]. Indeed, a newly developed seasonal allergy vaccine (Pollinex Quattro, Allergy Therapeutics, UK) formulated with MPL and grass or tree pollen has proved to be efficient in patients with seasonal allergic rhinitis [53]. Moreover, γ -PGA-stimulated murine DCs has recently been shown to regulate polarization of naïve CD4⁺ T cells towards Th1 cells over Th2 cells in a TLR4-dependent manner [44]. The present observations suggest that the capability of γ -PGA NPs to stimulate DCs through TLR2 and TLR4 may be beneficial in allergen-specific immunotherapy.

The mechanism behind γ -PGA NPs interaction with TLRs is not yet elucidated, but it is probable that hydrophobic regions of γ -PGA NPs function as danger signal and trigger TLR activation. TLR2 and TLR4 bind to a variety of structurally different ligands and many of these possess hydrophobic regions [54]. This is in accordance with preliminary findings suggesting that the immunostimulatory potential of NPs increases with hydrophobicity (Broos et al., unpubl.) and with previous studies on amphiphilic copolymers [55].

To determine the functional outcome of stimulation with γ -PGA NPs during allergen-presentation, we co-cultured allergen-stimulated MoDCs from grass pollen-allergic subjects with autologous memory T cells *in vitro* and assessed alterations in allergen-specific T cell proliferation and cytokine production. We demonstrated that γ -PGA NPs augmented allergen-specific proliferation of memory T cells. Interestingly, this was not accompanied with increased production of the Th2 cytokines IL-4, IL-5 and IL-13, or the Th1 or Th17 cytokines IFN- γ and IL-17. In contrast, a significant increase in IL-10 production was revealed, suggesting exclusive amplification of IL-10-producing memory T cells. Meiler et al. recently reported a switch from allergen-specific Th2 and Th1 cells *in vivo* towards IL-10 secreting type 1 regulatory T (Tr1) cells in bee keepers only a few days after exposure to high doses of bee venom antigen [56]. However, further studies are necessary in order to determine the exact nature of the T cells subsets present in our samples. Taken together, these findings, which resemble features of successful allergen-specific immunotherapy, can be produced by the employment of γ -PGA NPs.

In conclusion, we show that 200-nm sized γ -PGA NPs, which may specifically target myeloid DCs, induce immunomodulatory mediators that influence the Th1/Treg/Th2 balance. Notably, these NPs enhance proliferation and IL-10 production by allergen-specific T memory cells. Moreover, they stimulate DCs through TLR2 and TLR4, leading to expression of a wide range of genes involved in immune functions and efficient T cell priming. Taken together, the immunological effects of 200-nm sized γ -PGA NPs make them

promising as combined adjuvant and allergen-delivery system relevant to allergen-specific immunotherapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.05.004.

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