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Challenges and opportunities in nasal subunit vaccine delivery : mechanistic studies using ovalbumin as a model antigen

Slütter, B.A.

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5

Nanoparticles differentially modulate the outcome of nasal vaccination by enhancing mucosal tolerance or inducing protective immunity

Chantal Keijzer
Wim Jiskoot
Willem van Eden

Bram Slütter
Ruurd van der Zee
Femke Broere

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Abstract

In recent years, biocompatible and biodegradable polymeric nanoparticles have gained interest as antigen delivery systems. We investigated whether antigen-encapsulated PLGA (poly-Lactic-co-Glycolic Acid), PLGA-TMC (N-Trimethyl Chitosan) and TMC-TPP (Tri-Poly-Phosphate) nanoparticles can be used to modulate the immunological outcome towards active immunity or mucosal tolerance after nasal application.

The model protein ovalbumin (OVA) was encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles to explore induction of the antigen-specific B cell mediated humoral response and CD4⁺ T cell mediated responses after nasal application in a BALB/c mouse model.

We have demonstrated that nanoparticles enhanced the antigen presentation capacity of dendritic cells as shown by increased in vitro CD4⁺ T cell proliferation. We showed that nasal vaccination with low-dose OVA-encapsulated nanoparticles enhanced CD4⁺ T cell proliferation in contrast to low-dose sOVA treatment and that this coincided with enhanced FoxP3 expression in the NALT and CLN only when PLGA encapsulated OVA was applied. In nasal prime boost vaccination studies we showed that only with TMC-TPP treatment a humoral immune response was induced, which coincided with the enhanced generation of OVA-specific B cells in the CLN. Finally, in an OVA-specific DTH-model, nasal vaccination with PLGA nanoparticles induced mucosal tolerance as revealed by decreased levels in ear swelling at 24 h post challenge.

We have uncovered a role for nanoparticles to differentially direct the nasal mucosal immune response, towards B cell mediated protective immunity or towards CD4⁺ T cell mediated mucosal tolerance. The exploitation of this differential regulation capacity of nanoparticles to guide the immune response towards active or tolerogenic responses can lead to innovative vaccine development for prophylactic vaccination required in infectious diseases and for therapeutic vaccination in autoimmune diseases.

Introduction

Nasal vaccination is mainly described for the prevention of infectious diseases such as hepatitis B [1, 2] or influenza [3, 4]. However, in recent years, nasal application of antigen has become of interest in therapeutic interventions in the field of autoimmunity [5-8] and allergies [9]. Similar to other forms of mucosal immunization, such as oral immunization, nasal antigen application can stimulate antigen-specific responses locally and in the peripheral mucosal tissues [10-15]. Vaccination via the nasal mucosa might be preferred over oral vaccination due to the lower proteolytic activity at the nasal mucosa; this route of immunization requires a lower dose of antigen than for example oral immunization. Simultaneously, low antigen exposure might also reduce the chance of developing side-effects [16]. In general, mucosal antigen application can elicit protective immunity and/or a state of immunologic unresponsiveness, also termed antigen-specific mucosal tolerance [17-19]. Therefore, also nasal vaccination may divert immune responses to either activation of a protective antibody and/or T cell response desired during conventional vaccination or it may induce immunological tolerance desired as therapeutic treatment of autoimmune diseases.

The effectiveness of a nasal vaccine depends largely on the uptake of antigen by the nasopharynx-associated lymphoid tissue (NALT) [10, 11, 14, 15]. Since antigens are known to be more immunogenic in particulate form than in soluble form, advanced vaccine delivery systems are being developed, that specifically target NALT epithelium to enhance mucosal immunity [3, 13, 16, 20]. Nanoparticles are an example of such delivery systems and seem to be promising candidates for nasal vaccination due to their non-toxic characteristics [20-22].

In recent years, several *in vivo* studies have been conducted to investigate the additive role of nanoparticle mediated enhanced delivery of antigen at mucosal sites. The readout to evaluate the effectiveness of the applied vaccine relied mostly on induction of humoral responses as indicated by increased antigen-specific antibody titers [3, 23, 24]. However, it does not give insight in the underlying immunological mechanism that drives the response towards active immunity or tolerance induction. In addition, little is known about the role of CD4⁺ T cells in nasal vaccination and how nanoparticle treatment might influence the activation of these cells, locally and in the peripheral tissues. Therefore, we set out to understand how we can direct the induced response towards active immunity or tolerance through nasal nanoparticle delivery. In general, it is accepted that the induced response following mucosal antigen application depends on many factors such as the nature of the antigen (soluble versus particulate), antigen dose, size and delivery to the mucosal tissues as shown by previously conducted studies. For example, nasal application of TMC-TPP particles is

known to elicit a more active immune response as described by Amidi *et al* [3]. In this study, mice received three successive intranasal treatments with 3 weeks interval of TMC-TPP containing monovalent H3N2 influenza antigen particles with an average size of 800 nm. Treated mice showed a significant increase in antigen-specific immune responses as shown by an increase in antigen-specific IgG1/IgG2a serum titers and increased IgA titers in nasal washes [3].

In contrast to induction of active immunity, a study conducted by Kim Wan-Uk *et al* [23] showed that mice fed with a single dose of 40 µg of type II collagen (CII)-containing PLGA particles, with an average size of 300 nm, had reduced severity of arthritis and reduced anti-CII-specific IgG antibody titers and CII-specific T cell responses.

As mentioned before, the nasal route of antigen delivery has some advantages compared to oral application. Since this route of immunization requires a lower dose of antigen due to the low proteolytic activity locally, it might also reduce the chance of developing side-effects [16]. This could be of great benefit in the treatment of ongoing chronic inflammatory diseases, such as rheumatoid arthritis.

To study the mechanisms behind active immunity or tolerance after nasal vaccination in more detail, we chose three polymeric nanoparticles that have previously shown to provoke active immunity or tolerance after nasal administration; PLGA (poly-lactic-co-glycolic acid) [25, 26], PLGA-TMC (N-trimethyl chitosan) [27] and TMC-TPP (tri-polyphosphate) [28, 29] nanoparticles that all contained the model antigen ovalbumin (OVA). These particles have a similar average diameter, but differ in their surface charge and antigen release kinetics [24]. We investigated whether these nanoparticles can shift the immunological outcome towards active immunity or tolerance after nasal antigen application. More specifically, we have explored the effect of nasal application of OVA-encapsulated nanoparticles compared to soluble OVA delivery. More insight in the mechanism by which nanoparticles drive the immune response towards tolerogenic or protective responses will assist future rational vaccine design not only to prevent infectious diseases but also for therapeutic vaccination in autoimmune diseases.

Materials and Methods

Mice

Male BALB/c mice (8-12 weeks) were purchased from Charles River Laboratories (Maastricht, The Netherlands). OVA-specific TCR transgenic (Tg) mice on BALB/c background (DO11.10 mice) [30], were bred at the Central Animal Laboratory (GDL), Utrecht University,

the Netherlands. All mice were kept in our animal facility under routine laboratory conditions. Experiments were approved by the Animal Experiment Committee of the Utrecht University (Utrecht, The Netherlands).

Antibodies, antigens and OVA encapsulated nanoparticles

In all *in vitro* and *in vivo* experiments, intact 98% pure OVA (either from Sigma Aldrich (Zwijndrecht, The Netherlands) or from Calbiochem (San Diego, CA) was used. OVA-encapsulated PLGA, PLGA-TMC and TMC-TPP nanoparticles were generated as described previously [24]. The anti-clonotypic mAb for the DO11.10 Tg TCR (KJ1.26) was purified from culture supernatant and biotinylated, according to the manufacturer's protocol (Molecular Probes, Leiden, The Netherlands). 7-Amino-actinomycin-D (7-AAD)-unconjugated, Anti-CD11c (HL3), anti-CD4 (RM4-5), anti-CD40 (3/23), anti-CD86 (GL1), anti-MHC class II (M5/114), anti-CD25 (PC61) and anti-CD69 (H1.2F3) antibodies were purchased from BD Pharmingen (Woerden, The Netherlands). Anti-FoxP3-PE (FJK-16s) and an appropriate isotype control were purchased from eBioscience (Breda, The Netherlands).

DC isolation and culture

Bone marrow-derived dendritic cells (BMDC) were cultured from BALB/c donor mice as previously described by (Lutz 1999) with minor modifications. Briefly, on day 0, femurs and tibia of adult BALB/c mice were flushed with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Invitrogen) that contained heat-inactivated 10% FCS (Bodinco). Single cell suspensions were seeded at 3×10^6 per petri dish in complete medium with 20 ng/ml murine rGM-CSF (Cytogen). On day 2 and 4, 10 ng/ml murine rGM-CSF was added. On day 7, the BMDCs were harvested and used for further experiments.

CD4⁺ T cell enrichment and CFSE labeling

Spleens were isolated from DO11.10 donor mice and were prepared into single cell suspensions. Erythrocytes were lysed and CD4⁺ T cells were obtained by negative selection with sheep-anti-rat IgG Dynabeads (Dyna, Invitrogen, Breda, the Netherlands) using an excess amount of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114), anti-CD8 (YTS169) mAbs. Enriched CD4⁺ T cells were routinely pure between 85 and 90%. Labeling of cells with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) was performed as previously described by Broere et al. [31].

In vitro effect of nanoparticles on BMDC maturation, antigen uptake and T cell activation

To address direct maturation of BMDC by nanoparticles, BMDC were cultured in the presence of OVA containing PLGA, PLGA-TMC or TMC-TPP nanoparticles (25 ng/ml to 1 µg/ml), or 10 ng/ml LPS (Sigma) as a maturation control. After 24 h, DC maturation was determined by flow cytometry (FACS-Calibur; BD Pharmingen) and FlowJo Software V8.8.6.

BMDCs were incubated for 1.5 hours at either 4°C or 37°C with FITC-labeled OVA protein purchased from Molecular probes (Invitrogen, Breda, the Netherlands) solved in saline or incorporated into PLGA, PLGA-TMC or TMC-TPP. To quench external FITC, trypan blue stain (Gibco, Invitrogen) was added to each sample 5 minutes before FACS analysis at a final concentration of 0.02%. OVA-FITC uptake by BMDCs was analyzed by flow cytometry. BMDCs were pre-incubated at 37°C for 2 h in the presence of OVA protein solved in saline or OVA-nanoparticles; PLGA, PLGA-TMC or TMC-TPP at concentrations of 25 ng/ml, 0.5 µg/ml or 1 µg/ml. OVA-specific CD4⁺ T cells were added at a 1:10 ratio and T cell proliferation was assessed at 72 h post culture by CFSE dilution.

T cell activation in the local lymph nodes after nanoparticle vaccination

BALB/c acceptor mice were adoptively transferred with $1 \cdot 10^7$ CFSE-labeled CD4⁺KJ1.26⁺ cells in 100 µl saline, intravenously (i.v.) injected via the lateral tail vein. The next day mice received either a single application of 30 µg of OVA i.n. solved in 10 µl of saline or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles, or a single immunization of 30 µg of OVA intramuscularly (i.m.) in the hind limbs solved in 50 µl of saline or encapsulated in nanoparticles. At 72 h post i.n. or i.m. OVA administration, the spleen, the nose-draining NALT [11, 32] and cervical lymph nodes (CLN) as well as the thigh-draining inguinal lymph nodes (ILN) were removed and single cell suspensions were analyzed to evaluate *in vivo* T cell division by flow cytometry as described earlier.

Nasal prime-boost vaccination

BALB/c mice received three nasal applications of 20 µg of OVA i.n. dissolved in 10 µl of saline or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles, with a 3 week interval. Three weeks after third OVA vaccination, the spleen, NALT and CLN were removed and single cell suspensions were analyzed to evaluate OVA-specific T and B cell responses. Single cell suspensions were restimulated with OVA protein at final concentrations of 100 µg/ml for a 72 h period and 0.4 µCi ³H-thymidine (Amersham Health, Little Chalfont, Buckinghamshire, UK) was added for an additional 18 h to address proliferation. Supernatants were analyzed for cytokine production.

The *in vivo* B cell response was assessed by detection of OVA-specific antibody titers in serum of immunized mice as described elsewhere [24] by ELISPOT. Single cell suspensions from the NALT, CLN and spleen were cultured with OVA (1 µg/well) or control high protein binding filter plates (MultiScreen-IP, Millipore) for 48 hours. After incubation, SFC were detected with goat- anti mouse IgG-biotin (Sigma), Avidin-AP (Sigma). Plates were developed with NBT-BCIP (Roche) and analyzed by using the Aelvis spotreader and software. Data are shown as the net OVA-specific B cell count per 1×10^6 cells calculated as background (spots medium coated plates) subtracted from OVA-specific spots.

Mucosal tolerance induction and delayed-type hypersensitivity (DTH) reaction Balb/c mice received 20 µg of OVA i.n. three times at 24 h intervals either dissolved in saline or encapsulated in PLGA, PLGA-TMC or TMC-TPP nanoparticles. Control groups received saline alone or OVA at a final concentration of 300 µg in saline. Mice were sensitized for a DTH the next day with 100 µg of OVA in 25 µl of saline, mixed with 25 µl of IFA (Difco, BD. Alphen a/d Rijn, The Netherlands) subcutaneously (s.c.) administered in the tail base. Five days later, directly before challenge, the initial thickness of both ears was measured with an engineer's micrometer (Mitutoyo, Tokio, Japan). Subsequently, mice were challenged with 10 µg of OVA in 10 µl of saline given in the auricle of each ear and 24 h post-challenge, increase in ear thickness of both ears was determined. In all experiments the ear thickness was measured in a blinded fashion.

Luminex

The amount of cytokine secreted during a 72 h re-stimulation period was assessed by analyzing the culture supernatants. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IFN-γ (AN18), IL-2 (JES6-1A12), IL-4 (BVD4-1D11), IL-5 (TRFK5), IL-6 (MP5-20F3), IL-10 (JES5-2A5), IL12p70 (9A5), IL-17A (TC11-18H10) and TNF-α (G281-2626)(BD Biosciences Pharmingen) were added to 50 µl of culture supernatant. Cytokines were detected by biotinylated antibodies IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BVD6-24G2), IL-5(TRFK5), IL-6 (MP5-32C11), IL-10 (SXC-1), IL12p70 (C17.8), IL-17(DuoSet ELISA kit, R&D systems Europe Ltd, Oxon, the U.K.) and TNF-α (MP6-XT3)(BD Biosciences Pharmingen) and PE-labeled streptavidin. Fluorescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA).

Real-time PCR

Total mRNA was purified from single cell suspensions from NALT, CLN, ILN or spleen using the RNeasy kit (Qiagen Benelux B.V.) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, B.V.) according to the manufacturer's protocol. Real-time quantitative PCR was performed using a MyiQ Single-Color Real-Time PCR detection system (Bio-Rad Laboratories B.V.) based on specific primers and general fluorescence detection with SYBR Green (iQ SYBR Green Supermix, Bio-Rad laboratories, Hercules, CA). Conditions for the Real-time quantitative reaction were (95°C for 3 min and 40 cycles of 95°C for 10 s and 59.5°C for 45 s). Expression was normalized to the detected Ct values of hypoxanthine-guanine phosphoribosyltransferase (HPRT) for each sample. The expression levels relative to HPRT were calculated according to the Real-Time PCR Bio-Rad manual by following the equation: relative expression level = $2^{-\Delta\Delta Ct}$ (Livak Method).

Specific primers were designed across different constant region exons resulting in the following primers:

HPRT sense 5'-CTGGTGAAAAGGACCTCTCG-3', antisense 5'- TGAAGTACTCATTATAGTC AAGGGCA-3'. **IL-10** sense 5'- GGTTGCCAAGCCTTATCGGA-3', antisense 5'- ACCTGCTCC ACTGCCTTGCT-3'. **FoxP3** sense 5'- CCCAGGAAAGACAGCAACCTT-3', antisense 5'-TTCT CACAACCAGGCCACTTG-3'. **IL-4** sense 5'- GGTCTCAACCCCCAGCTAGT-3', antisense 5'- GCCGATGATCTCTCTCAAGTGAT-3'. **IL-17** sense 5'- GCTCCAGAAGGCCCTCAGA-3', anti sense 5'- AGCTTTCCCTCCGCATTGA-3'. **IFN- γ** sense 5'- TCAAGTGGCATAGATGTGGAAG AA-3', antisense 5'- TGGCTCTGCAGGATTTTCATG-3'. **T-BET** sense 5'- CAACAACCCCTTT GCCAAAG-3', antisense 5'- TCCCCCAAGCAGTTGACAGT-3'. **GATA-3** sense 5'- AGAACCG GCCCCTTATCAA-3', antisense 5'- AGTTCGCGCAGGATGTCC-3'.

Statistics

Statistical analysis was performed with Prism software (Graphpad Software Inc., San Diego, version 4.00) using an unpaired two-tailed Student's *t* test, a one-way ANOVA followed by Tukey's multiple comparison test or by Dunn's multiple comparison test or Bonferroni's multiple comparison test. Error bars represent the SEM as indicated. Statistical differences for the mean values are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Differential uptake of OVA labeled FITC by BMDCs after in vitro nanoparticle treatment

From previous studies we know that nanoparticles show differences in localization after DC encounter as visualized by tracing uptake of OVA [20]. Based on these results we hypothesized that these differences in localization might modulate the subsequent antigen presentation capacity of DCs.

To investigate this hypothesis, we treated DCs *in vitro* with OVA encapsulated PLGA, PLGA-TMC or TMC-TPP nanoparticles or soluble OVA (sOVA) as a control and studied phenotypic and functional differences. First we studied whether nanoparticle treatment has an effect on DC maturation, viability and differentiation. The cells were stained for CD11c⁺, MHC-class-II⁺, and 7-AAD⁻ and analyzed for their expression of CD40 and CD86. We did not observe differences in DC viability or maturation after nanoparticle treatment at OVA concentrations varying from 1 ng/ml to 1 µg/ml. Furthermore, we could not detect significant differences in culture supernatants that were analyzed for cytokine secretion of TNF-α, IL-12p70, IL-4, IL-5 (**data not shown**).

Next, we studied the uptake of the PLGA, PLGA-TMC and TMC-TPP nanoparticles with OVA labeled FITC. Then the cells were stained for CD11c and MHC-class-II and uptake of OVA-FITC was assessed by flow cytometric analysis after silencing extracellular FITC signaling with trypan blue. OVA-FITC association with DCs is shown as the FITC ΔMFI expression (figure **1A**) or percentage of OVA-FITC positive cells (figure **1B**).

OVA-FITC uptake by DCs treated with TMC-TPP was lower compared to soluble OVA (sOVA)-FITC treatment as shown by a low FITC ΔMFI expression and decreased percentage of OVA-FITC positive cells (figure **1A-B**). Furthermore, compared to sOVA, PLGA-TMC treatment enhanced the antigen uptake by DCs even at low (25 ng/ml) OVA concentrations. Both PLGA and PLGA-TMC treatment enhanced antigen uptake was observed with OVA at 0.25 µg/ml. We could not detect differences in antigen uptake at 1.00 µg/ml sOVA, PLGA or PLGA-TMC treatment suggesting a maximum antigen uptake after 1.5 h of incubation (figure **1B**).

In summary, nanoparticle characteristics affected the antigen uptake by DCs *in vitro* as shown by a lower number of OVA-FITC positive cells when DCs encounter TMC-TPP particles compared to PLGA and PLGA-TMC.

OVA-encapsulated nanoparticles enhance OVA-specific CD4⁺ T cell proliferation in vitro

To investigate whether enhanced antigen uptake by DCs also affects the antigen presentation capacity of DCs, nanoparticle treated cells were studied *in vitro* by co-culture assay. DCs treated with OVA encapsulated PLGA, PLGA-TMC or TMC-TPP particles were cultured in the presence of OVA-specific CFSE-labeled CD4⁺ T cells isolated from spleen of DO11.10 mice. T cell proliferation as measured by CFSE dilution served as readout for antigen presentation efficiency of DCs.

Antigen presentation capacity of DCs was significantly enhanced after nanoparticle treatment in contrast to sOVA since T cells stimulated by particle treated DCs showed enhanced T cell proliferation compared to T cells cultured in the presence of sOVA treated DCs. Especially, PLGA and PLGA-TMC particles potently enhanced CD4⁺ T cell proliferation even at a low OVA concentration of 25 ng/ml (figure **1C**). Additionally, in the culture supernatants of T cells stimulated in the presence of 1.0 µg/ml OVA containing PLGA or PLGA-TMC particles more IL-2, IFN-γ and IL-10 was detected compared to cultures with TMC-TPP particles or sOVA (figure **1D**).

In conclusion, all three OVA loaded nanoparticles enhanced the antigen presentation by DCs, as shown by increased CD4⁺ T cell proliferation profiles as compared to sOVA.

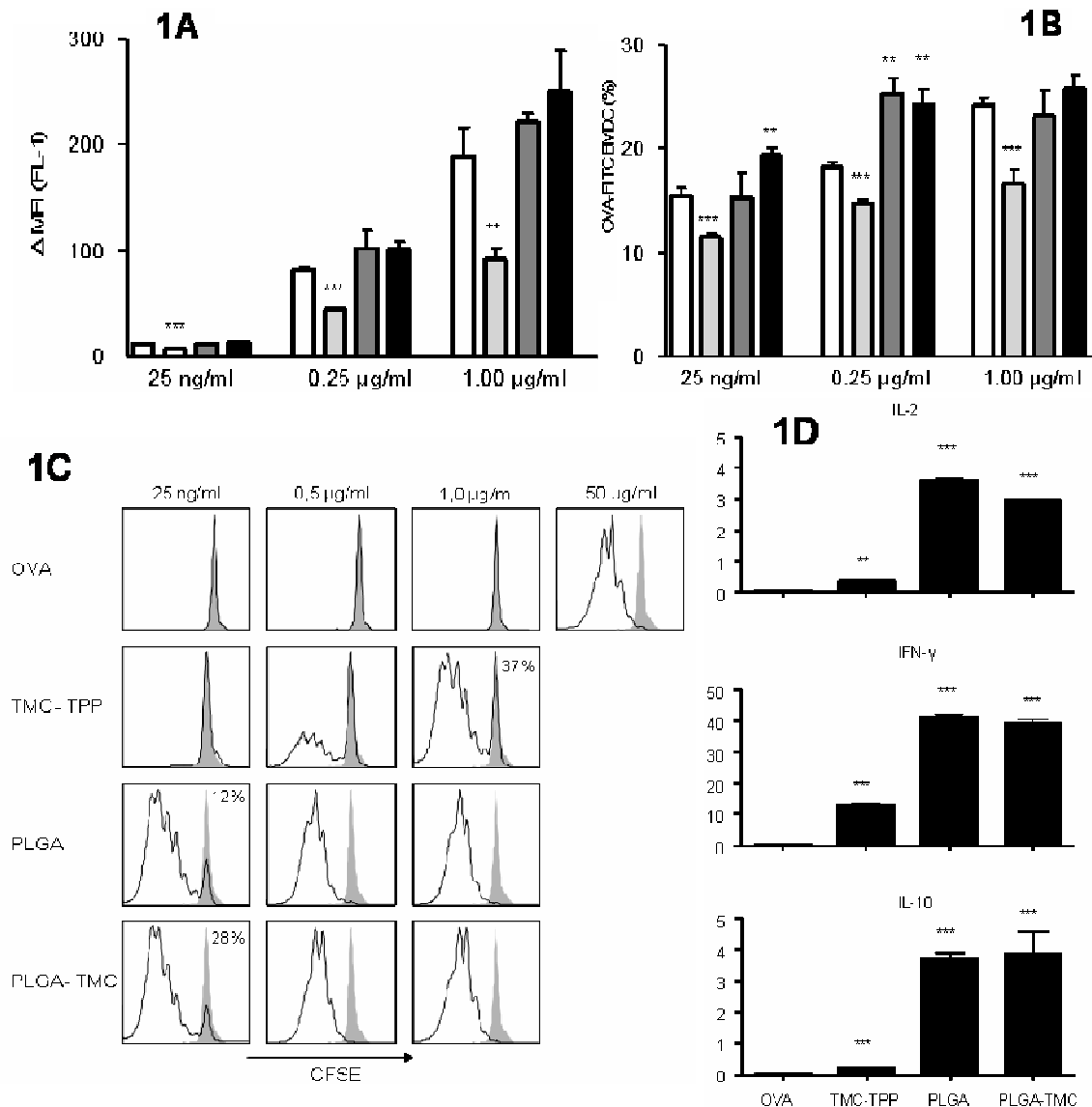


Figure 1: Nanoparticle mediated enhanced antigen presentation capacity of BMDCs in vitro. BMDC were incubated in the presence of sOVA-FITC or OVA-FITC encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles at different concentrations. External FITC signaling was silenced by trypan blue. The Δ MFI of OVA-FITC was assessed by subtraction of FITC signaling at 4°C from 37°C (**1A**). OVA-FITC uptake by BMDC shown as the percentage of OVA-FITC positive cells 1.5 h post-culture were calculated as the percentage at 4°C subtracted from the percentages at 37°C (**1B**). Data are representative for 3 independent experiments. mean $\pm$ standard error of the mean (s.e.m.). BMDCs were cultured in the presence of sOVA or OVA encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles at different concentrations and OVA-specific CFSE-labeled CD4⁺ T cells Gray filled histograms; unstimulated CD4⁺ T cells, Black overlays; CD4⁺ T cell division patterns at different OVA concentrations after 72 hours (**1C**). Data are are representative for at least 3 independent experiments. Cytokine concentrations of IL-2, IFN- γ and IL-10 (ng/ml) were determined in culture supernatants, after 72 h of culture (**1D**). Data are representative for 3 independent experiments. mean $\pm$ s.e.m. Statistically significant ** ($P < 0.01$), *** ($P < 0.001$).

Enhanced OVA-specific CD4⁺ T cell proliferation at mucosal (nasal) and non-mucosal (intramuscular) sites and enhanced local mRNA expression of FoxP3 after nanoparticle vaccination

Next, we questioned whether nanoparticle treatment also affects T cell response *in vivo*. We know from former studies that especially TMC-TPP nanoparticles induced increased generation of antigen-specific IgG1 and IgG2a antibody titers after both i.n. and i.m. vaccination, whereas PLGA and PLGA-TMC only resulted in higher IgG titers after i.m. vaccination and had little effect on the humoral immune response after i.n. treatment [24]. Here, we explored whether nanoparticle treatment affects the CD4⁺ T cell response and how this depends on nanoparticle type. First we studied the short-term CD4⁺ T cell response in mice that were either treated i.n. or i.m. with 30 µg of sOVA or OVA encapsulated particles. Proliferation of OVA-specific CFSE-labeled CD4⁺ T cells was addressed locally in the draining lymph nodes as well as systemic in the spleen at 72 h post treatment (figure **2A**).

Nasal vaccination enhanced local CD4⁺ T cell proliferation in the NALT and CLN compared to sOVA treatment, irrespective of the type of nanoparticles. However, none of the formulations induced measurable CD4⁺ T cell activation in the spleen at 72 hours after vaccination upon i.n. immunization (figure **2A; left**). In contrast, non-mucosal vaccination resulted in proliferation both in the draining ILN and spleen at this time point (figure **2A; right**).

We could not detect significant differences in cytokine profiles in culture supernatants of the isolated draining CLN and ILN organs after particle vaccination (**data not shown**). However, we observed a significant increase in the relative FoxP3 mRNA expression in the CLN and a slightly increased expression in the NALT of mice that had received a single i.n. PLGA vaccination (figure **2B**). Mice that were vaccinated i.m. with TMC-TPP particles showed less expression of FoxP3 mRNA compared to PLGA and PLGA-TMC treated mice.

These data show that i.n. vaccination with low-dose OVA encapsulated nanoparticles enhanced CD4⁺ T cell proliferation in contrast to low-dose sOVA treatment (**2A**) and coincided with enhanced FoxP3 in the NALT and CLN only when PLGA encapsulated OVA was applied. This effect was lacking in the i.m. treated mice in all treatment groups (figure **2B**) showing that both particle and route of application determine the outcome of the CD4⁺ T cell response. Although induced CD4⁺ T cell proliferation mediated by TMC-TPP was less efficient compared to PLGA and PLGA-TMC treatment *in vitro* (figure **1C**), we were not able to detect such significant differences in CD4⁺ T cell proliferation profiles *in vivo* (figure **2A**).

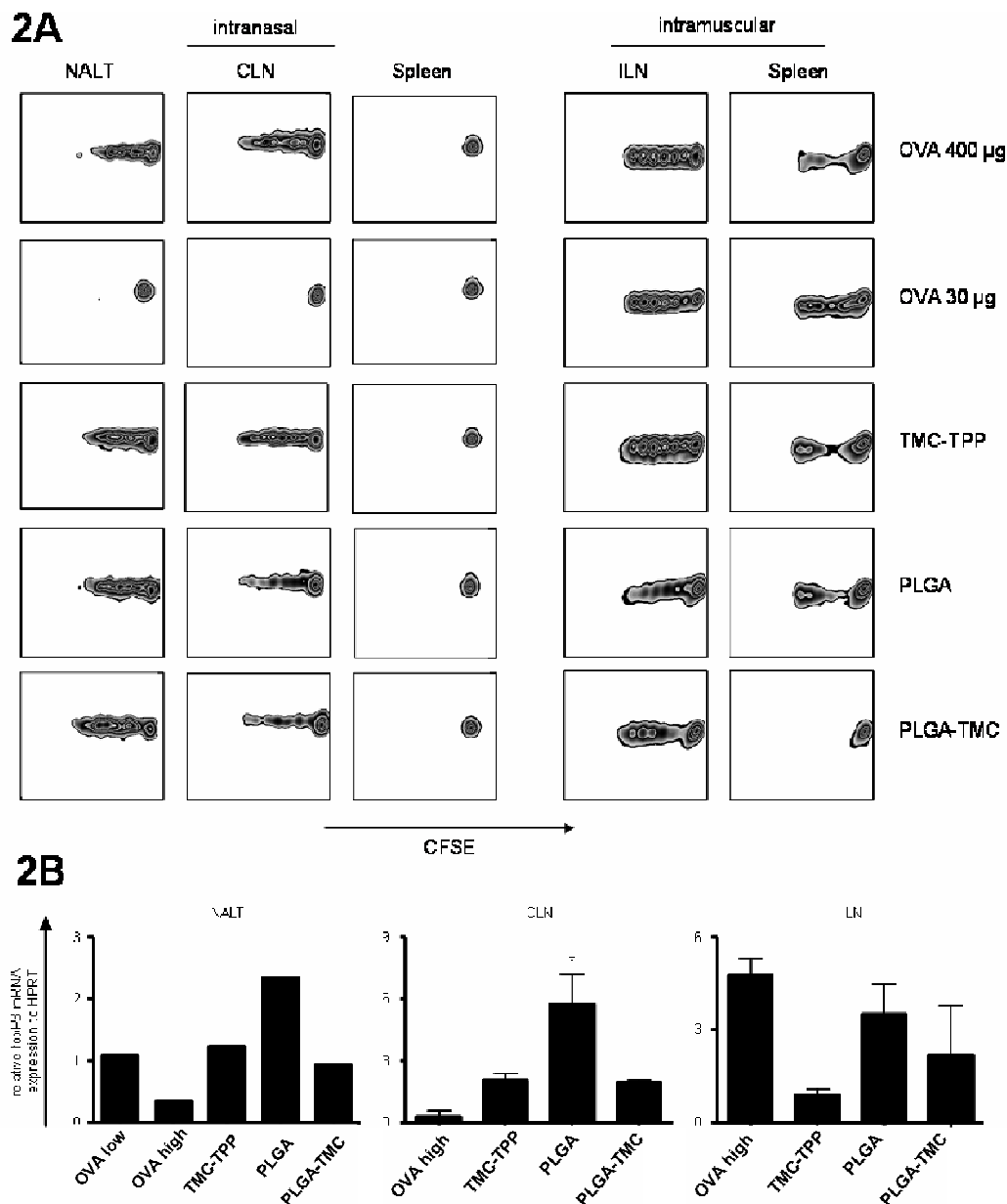


Figure 2: Enhanced OVA-specific CD4⁺ T cell proliferation at mucosal and non-mucosal sites, after nanoparticle treatment. OVA-specific CFSE labeled CD4⁺ T cells were transferred to BALB/c acceptor mice one day prior to vaccination. Mice received a single i.n. application of 30 µg of sOVA or OVA encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles. For induction of a non-mucosal response, mice received a single i.m. immunization in the hind limbs. At 72 h post i.n. or i.m. OVA administration, in vivo T cell division was addressed in spleen, nose-draining NALT and CLN as well as the thigh-draining ILN (**2A**). Data are representative for at least 3 (intranasal) and 2 (intramuscular) independent transfer studies. Total mRNA was purified from single cell suspensions from *NALT, CLN, and ILN. Relative mRNA expression to HPRT of FoxP3 was determined 72 h post i.n. or i.m. OVA application (**2B**). NALT: intranasal treatment (cells isolated from NALT were pooled per group). CLN: PLGA (n=5); PLGA-TMC (n=3); TMC-TPP (n=4) and ILN (n=3 mice per group); mean ± s.e.m. Statistically significant: * (P< 0.05).

OVA-specific CD4⁺ T cell and B cell response after nasal prime-boost vaccination

In the previous study we explored the differences in the induction of an OVA-specific CD4⁺ T cell response in a T cell transfer study. The effect of differences in antigen delivery (soluble versus particulate), antigen dose (low or high) and route of administration (i.n. or i.m.) was assessed by comparing the induced CD4⁺ T cell response 72 h post nasal vaccination. We were able to detect a significant difference in the enhanced CD4⁺ T cell proliferation profile 72 h post nasal vaccination of mice that received a single low-dose of OVA encapsulated PLGA, PLGA-TMC or TMC-TPP nanoparticles in contrast to sOVA treatment (figure **2A**). In addition, the local CD4⁺ T cell response seemed to be specifically shifted to a regulatory response upon i.n. PLGA treatment as shown by enhanced expression of the relative mRNA FoxP3 expression in these mice (figure **2B**). As mentioned before, in a previous study we described that especially i.n. TMC-TPP vaccination induced enhanced generation of antigen-specific IgG1 and IgG2a antibody titers while PLGA and PLGA-TMC had hardly any effect on the induced humoral immune response [24].

We performed an additional vaccination study to further assess the effect of i.n. nanoparticle treatment on the induction of an OVA-specific T cell or B cell response. Briefly, mice received 20 µg of OVA i.n. three times at three week intervals. Three weeks after the final OVA application single cell suspensions from the spleen, NALT and CLN were analyzed to evaluate OVA-specific T and B cell responses by ³H-thymidine incorporation or ELISPOT, respectively. We could not detect significant differences in the T cell proliferation profiles of CLN and spleen after i.n. treatment. However, a slightly enhanced T cell proliferation profile was observed after PLGA vaccination in contrast to PLGA-TMC and TMC-TPP as indicated by the SI and cpm (figure **3A-B**).

Beside the T cell response we investigated if nanoparticle treatment had a differential effect on B cell stimulation. Here, results showed that after i.n. vaccination, both sOVA and TMC-TPP treatment slightly enhanced the generation of OVA specific B cells locally in the draining CLN as shown in figure (figure **3C**). Moreover, increased numbers of OVA-specific B cells were detected in the spleens of mice after TMC-TPP and PLGA-TMC vaccination (**data not shown**). These results correlate with the findings of increased generation of antigen-specific antibodies after nasal TMC-TPP vaccination as described by Slütter *et al* [24].

In summary, we were able to detect significant differences in the induced type of immune response after nanoparticle treatment. Nasal treatment with low-dose OVA encapsulated PLGA nanoparticles enhanced the CD4⁺ T cell response and relative FoxP3 mRNA expression locally in the NALT and CLN. Although TMC-TPP nanoparticles showed to be superior in the activation of the humoral arm of the nasal mucosal immune system as shown by increased

generation of OVA-specific B cells and OVA-specific antibody titers in serum and nasal washes, this effect was not observed for PLGA treated mice. From these data we hypothesized that nasal application of slow release PLGA nanoparticles induced T cell mediated mucosal tolerance whereas TMC-TPP showed to induce protective immunity upon i.n. vaccination. To further study if these differences are of functional importance, the nanoparticles we tested in an OVA-specific delayed-type hypersensitivity (DTH) model.

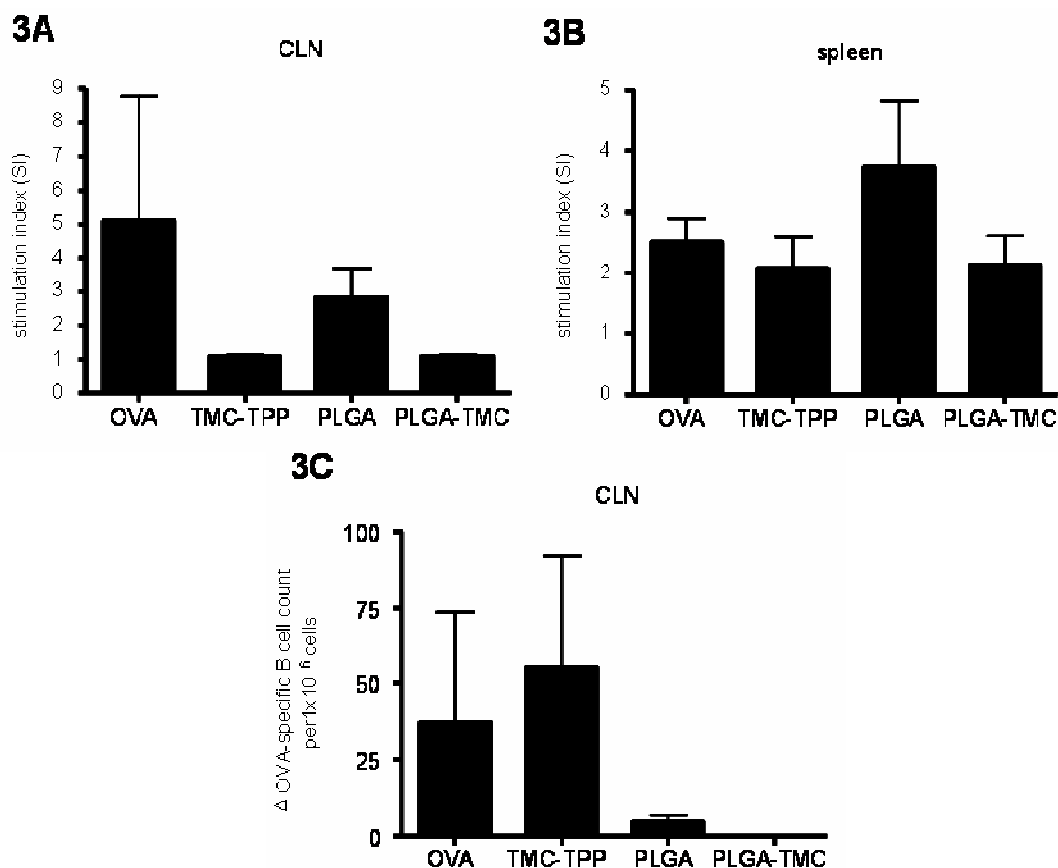


Figure 3: OVA-specific $CD4^+$ T cell and B cell response after nasal prime-boost vaccination. Effect of nanoparticles after nasal vaccination on OVA-specific T cells (**A-B**). Mice received three times $20 \mu\text{g}$ of OVA solved in PBS or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles. Three weeks post the final OVA administration, the OVA-specific T cell proliferation was assessed ex vivo. Single cell suspensions were re-stimulated for 72 h in the presence of OVA prior to ^3H -thymidine incorporation. Data are shown as the stimulation index (SI) of cells isolated from the CLN (**3A**) or spleen (**3B**). Effect of nanoparticles after nasal vaccination on OVA-specific B cells (**C**). Mice were treated as described above. Three weeks post the final OVA administration, the OVA-specific B cell response was assessed ex vivo 48 h post re-stimulation of single cell suspensions by ELISPOT. Data are shown as cells isolated from CLN (**3C**). Data are shown as the Δ OVA-specific B cell count per 1×10^6 cells calculated as background (spots counted on medium coated plates) subtract from OVA-specific spots. $n=5$ mice per group; mean $\pm$ standard error of the mean (s.e.m.).

Nasal application of slow-release PLGA particles suppressed a Th-1-mediated hypersensitivity reaction, while TMC-TPP enhanced humoral immunity, in an OVA-specific delayed-type hypersensitivity (DTH)-model

In general we showed that after nasal vaccination PLGA treatment induced a more T cell mediated immune response, whereas TMC-TPP particle treatment resulted in increased numbers of OVA-specific B cells. To see if these differences are of functional importance, the nanoparticles were tested in a DTH-model. After 24 h, changes in ear swelling were determined and compared with values prior to challenge. Clearly, PLGA nanoparticle treatment suppressed the OVA specific DTH response, whereas PLGA-TMC and TMC-TPP nanoparticle treated mice failed to suppress the DTH response 24 h post challenge (figure **4A**). In contrast to PLGA and PLGA-TMC, nasal application of TMC-TPP significantly increased the generation of OVA-specific B cells locally in the draining CLN (figure **4B**) and to a lower extent in the spleen (figure **4C**). This suggests a main role for TMC-TPP in the activation of the humoral immune response and agrees with earlier findings [24]. In addition, a significant increase in the relative IL-10 mRNA expression was detected in the CLN (figure **4D**), but not in the spleen (**data not shown**) of mice that were tolerized by PLGA vaccination. Although we could detect increased expression of relative FoxP3 mRNA in the T cell transfer study (figure **2B**) we were not able to detect such significant differences in the DTH model, probably due to experimental differences in timing and presence of OVA-specific T cells in draining lymph nodes (**data not shown**).

To summarize, nasal treatment with PLGA nanoparticles induced mucosal tolerance but did not induce protective immunity as shown by the absence of antigen-specific responses (figure **4A-C**). In contrast to PLGA and PLGA-TMC immunization, only TMC-TPP treatment led to activation of the humoral immune response as shown by local increased generation of antigen-specific B cells (figure **3C, 4B-C**) and increased levels of antigen-specific antibody titers systemically. We were not able to detect a clear T or B cell induced response upon nasal PLGA-TMC treatment (figure **3 and 4**). However, we can conclude that PLGA-TMC immune regulation was somewhere intermediate, since PLGA-TMC treatment only partially suppressed the DTH response (figure **4A**).

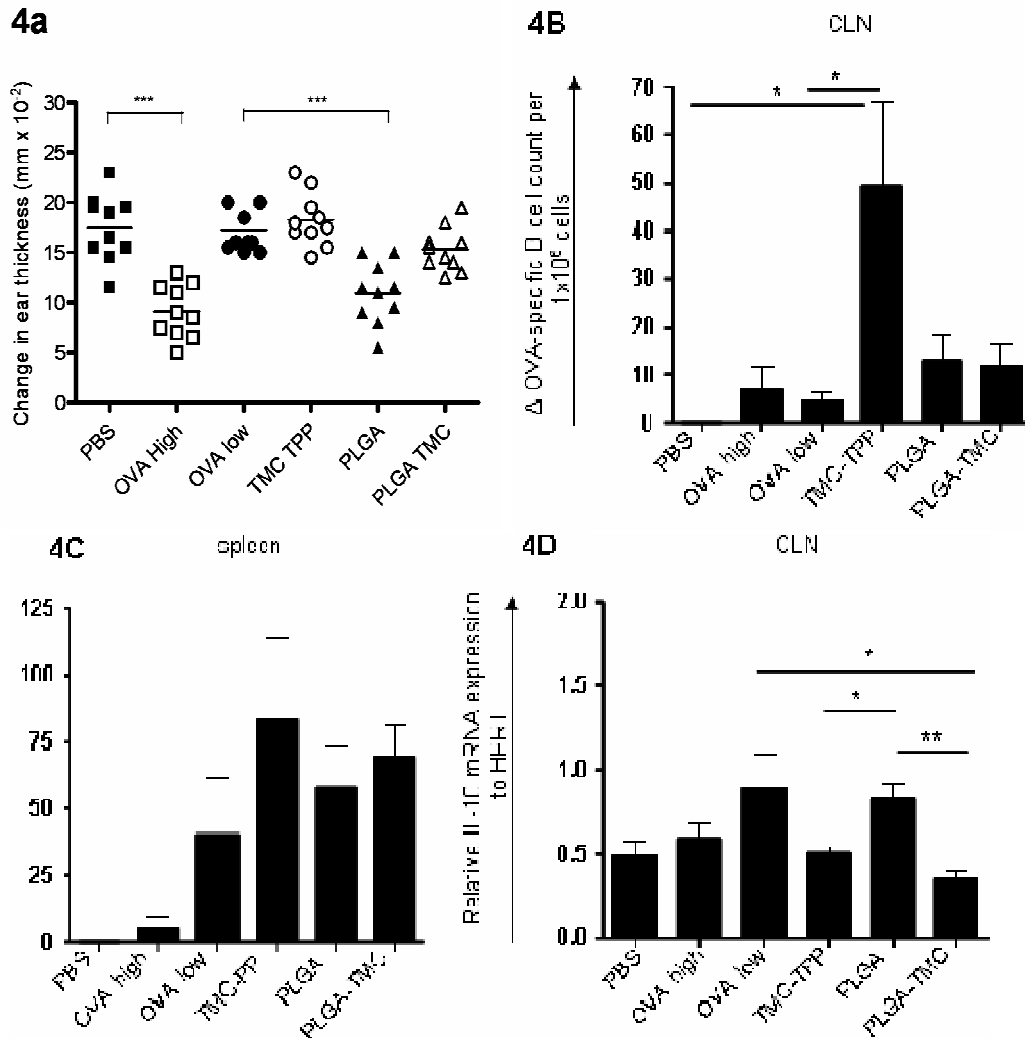


Figure 4: Low Nasal application of slow-release PLGA particles suppressed a Th-1-mediated hypersensitivity reaction, while TMC-TPP enhanced humoral immunity, in an OVA-specific DTH model. Effect of nanoparticles on nasally induced suppression of a DTH response in BALB/c mice (**4A**). Mice received 20 μ g of OVA i.n. either solved in PBS (black circles) or encapsulated in TMC-TPP (white circles), PLGA (black triangles) or PLGA-TMC (white triangles) nanoparticles for three successive days. Control mice were treated with PBS only (black squares) or with OVA at a concentration of 100 μ g (white squares). Mice were sensitized subcutaneously at the tail base with 100 μ g of OVA in IFA 1 day post the final nasal OVA administration. Five days after sensitization, mice were challenged with 10 μ g of OVA in 10 μ l of PBS in the auricle of both ears. After 24 h, changes in ear thickness were determined and compared with values before challenge. Enhanced OVA-specific B cell response induced after nasal nanoparticle treatment (**4B-C**). OVA-specific B cell response was assessed ex vivo 48 h post re-stimulation of single cell suspensions by ELISPOT. Data are shown as cells isolated from CLN (**4B**) and spleen (**4C**). Data are shown as the Δ OVA-specific B cell count per 1×10^6 cells calculated as background (spots counted on medium coated plates) subtract from OVA-specific spots. OVA-encapsulated PLGA nanoparticles enhance local mRNA expression of IL-10 after nasal vaccination (**4D**). Total mRNA was purified from single cell suspensions from the CLN and subsequently reverse transcribed into cDNA. Relative mRNA expression to HPRT of and IL-10 was determined 48 h post OVA challenge in the CLN (**4D**) of mice. $n=5$ mice per group; mean $\pm$ standard error of the mean (s.e.m.). One representative experiment of two is shown. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

Discussion

The nasal route is an attractive alternative for classical vaccination based on several specific characteristics of the mucosal tissue [16, 33]. Former studies already showed that upon nasal nanoparticle treatment the humoral immune response can be enhanced and that autoimmunity could be suppressed by mucosal tolerance induction [3, 23, 24]. Since there is not much known about the role of CD4⁺ T cells upon nasal nanoparticle treatment, we explored how nanoparticle treatment affected CD4⁺ T cell activation both *in vitro* and *in vivo*. In this study we investigated whether OVA-encapsulated nanoparticles can modulate the immunological outcome towards active immunity or tolerance after nasal antigen application.

We showed *in vitro* that particle characteristics influenced OVA-specific CD4⁺ T cell proliferation as shown in **figure 1C** by the absence of activated T cell after sOVA treatment. While PLGA and PLGA-TMC nanoparticles enhanced CD4⁺ T cell proliferation to a degree that all T cells had divided at least one time, we observed a relative high peak of undivided T cells when DCs were pulsed with TMC-TPP nanoparticles at similar concentrations, at 72 h post culture. The amount of antigen uptake does not necessarily correlate with the percentage of cell division since we observed a relative lower uptake of OVA-FITC in TMC-TPP pulsed DCs, whereas T cell proliferation was enhanced. One possible explanation can be that the differential CD4⁺ T cell proliferation profiles are caused by the diversity in how the nanoparticles encounter DCs. Previous studies have described significant differences in how nanoparticles interact with DCs *in vitro* [20]. TMC-TPP particles release the antigen by a mechanism of rapid content release. Since these particles easily associate with the outer cell wall [24, 34], antigen may be efficiently taken up by DCs in contrast to sOVA that is scattered throughout the entire culture supernatant. Although these particles are less efficiently taken up compared to PLGA and PLGA-TMC they may in time increase the antigen uptake by DCs as shown by enhanced CD4⁺ T cell proliferation *in vitro* in comparison with sOVA at 72 h post culture. We were not able to detect differences in DC maturation or phenotype suggesting that particle treatment mainly affects the antigen presentation capacity of DCs. Since direct evidence is lacking, the functional interaction of nanoparticles with DCs may receive further attention.

We showed that low-dose OVA encapsulated nanoparticles enhanced OVA-specific CD4⁺ T cell proliferation locally in the NALT and CLN after a single nasal application, which was not seen with low-dose sOVA. This showed the superiority of nanoparticle mediated OVA delivery versus sOVA delivery. Interestingly, due to the absence of activated OVA-specific T cells in the spleen following nasal treatment we can conclude that more time is required to elicit systemic

responses in comparison to non-mucosal antigen immunization (figure **2A**). Our data also confirm that the route of antigen delivery, mucosal versus non-mucosal, differentially activated the immune system as previously described by Unger *et al* [19].

It was already described that both protective immunity and mucosal tolerance induction could be enhanced after nasal nanoparticle treatment¹⁰. The differential role of nasal nanoparticle application in the activation of fundamental T and B cells function however had not been investigated. In a prime-boost vaccination study, we showed that nasal vaccination with low-dose OVA-encapsulated PLGA nanoparticles enhanced primarily T cell activation both in the local CLN and systemically in the spleen. Such enhanced T cell proliferation was not detected after TMC-TPP or PLGA-TMC application. In contrast to PLGA and PLGA-TMC, treatment with TMC-TPP led to increased generation of OVA-specific B cells locally in the CLN and this correlates with increased levels of antigen-specific antibody titers. Altogether, these data clearly show that nanoparticles differentially modulate the activation of T and B cells after nasal delivery.

From the literature it is known that CD4+CD25+FoxP3+ regulatory T cells play an important role in the induction of mucosal tolerance [35, 36]. Therefore, we decided to explore the expression of FoxP3 by T cells after nasal nanoparticle treatment. We were capable of detecting a significant difference in the mRNA FoxP3 expression levels between the nanoparticle treatment groups locally in the CLN. Interestingly, we observed an increased FoxP3 expression only for PLGA treatment (figure **2B**).

Finally, we studied the functional significance of these basic findings by testing the nanoparticles in a DTH-model. We found that when mice were tolerized for OVA by nasal treatment with low-dose OVA-encapsulated PLGA nanoparticles, a T cell mediated tolerogenic response was induced. This type of tolerance induction was lacking in TMC-TPP treated mice that again showed a significant increased generation of OVA-specific B cells in the CLN in contrast to the other treatment groups. Although we observed a significant increased expression of FoxP3 mRNA in mice treated with PLGA nanoparticles in the OVA-specific CD4+ T cell transfer studies, we were not able to detect such differences in FoxP3 expression levels in this experiment. However, in the T cell transfer studies, the number of OVA-specific T cells was enhanced as compared to the DTH model, making it less likely to detect these cells. In addition, transferred T cells in the transfer model were analyzed 72 h post transfer when OVA-specific T cells were still present locally in the draining lymph nodes as shown earlier in figure 2A. Compared to the transfer model, in the DTH experiment, T cells were analyzed 9 days after the last nasal immunization giving T cells enough time to enter the circulation and making it difficult to detect them locally. Although the subtype of T cell that mediated

tolerance remains unclear, we were able to observe a significant increased expression of IL-10 mRNA after PLGA treatment locally in the CLN (figure **4D**). Therefore we suggest that tolerance after nasal PLGA treatment may be IL-10 mediated.

In conclusion, our results indicate that nasal PLGA or TMC-TPP nanoparticles can shift the antigen-specific immune response to tolerance or active immunity, respectively. These findings may increase the possibility to use nanoparticles to drive the immune response towards tolerance or protective immunity and enable future rational vaccine design not only to prevent infectious diseases but also for therapeutic vaccination in autoimmune diseases.

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