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The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation

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ABSTRACT

Delivery of nucleic acids with positively charged lipid nanoparticles ((+)NPs) is widely used as research reagents and potentially for therapeutics due to their ability to deliver nucleic acids into the cell cytoplasm. However, in most reports little attention has been made to their toxic effects. In the present study, we performed comprehensive analyses of the potential toxicity associated with (+)NPs. Mice treated with (+)NPs showed increased liver enzyme release and body weight loss compared to mice treated with neutral or negatively charged NPs ((−)NPs), suggesting hepatotoxicity. Intravenous administration of (+)NPs induced interferon type I response and elevated mRNA levels of interferon responsive genes 15–25-fold higher than neutral and (−)NPs in different subsets of leukocytes. Moreover, treatment with (+)NPs provoked a dramatic pro-inflammatory response by inducing Th1 cytokines expression (IL-2, IFN γ and TNF α) 10–75-fold higher than treatment with control particles. Finally, we showed that activation of TLR4 might serve as the underlying mechanism for induction of an immune response when (+)NPs are used. These results suggest that a careful attention must be made when different types of (+)NPs are being developed as nanotherapeutics.

1. Introduction

Non-viral gene delivery vectors are biomaterials, which are often based on cationic components [1]. The nature, shape and charge of the biomaterials affect the interaction with the immune system. Several studies laid the foundation of investigating the immune response when developing novel biomaterials for gene transfer and those focused mainly on lymphocytes and complement activation [2,3] or examine global cell counts of subtypes of leukocytes when injecting novel formulations into animal models [4–6].

Tremendous efforts to develop carriers for nucleic acids delivery are made since the discovery of RNA interference (RNAi) in 1998 [7] and the application of small interfering RNAs (siRNAs) in mammalian cells in 2001 [8]. RNAi has emerged as a powerful tool for elucidating gene function, identifying novel drug targets and potentially utilizing it for therapeutic intervention [9,10]. The work by Kleinman et al. [11] demonstrated that siRNAs can activate the innate immunity via endothelial Toll-like receptor 3 (TLR3). This work emphasized the importance of understanding siRNA non-specific toxicity and directed major efforts to this task. Indeed, it has been revealed that siRNA are potent activators of the mammalian innate immunity, inducing inflammatory cytokines and interferon response mainly through RNA-sensing TLRs (TLR 3, 7 and 8) [12,13]. To overcome this hurdle, several chemical modifications and sequence preferences were developed successfully [14–16].

Due to several characteristics of siRNA such as its relatively large size (~14 kDa), its negative charge (~40 charge units/siRNA molecule) that cause poor cellular uptake and its susceptibility to enzymatic degradation in vivo, therapeutic applications of RNAi are heavily dependent on the development of appropriate carriers that can provide protection and transport the RNAi payload into cells of interest. Currently the rate-limiting factor in therapeutic RNAi is development of safe and efficient delivery vehicles. To this end, a variety of carriers utilizing both natural and synthetic materials have been developed [2,17–19]. Cationic lipids in general and DOTAP lipid in particular stand for one of the most well studied carriers for RNAi delivery. Positively charged nanoparticles ((+)NPs) composed of cationic lipids posses the ability to bind and condense siRNA through electrostatic interactions and to deliver the payload across the cellular membrane into the cytoplasm of target cells [20]. Although siRNA off-target effects have been widely

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studied, understanding the carrier side effects and toxicity is crucial and it is less characterized at the immune cellular level.

Herein, we report the analyses of the potential toxicity associated with positively charged NPs at the cellular level and propose the underlying mechanism.

2. Materials and methods

2.1. Materials

High-purity hydrogenated soy phosphatidylcholine (HSPC), and 1,2-distearoylphosphatidylglycerol (DSPG) were a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar lipids Inc. (Alabaster, AL, USA). Cell culture plates and dishes were from Corning Glass Works (New York, NY, USA). Polycarbonate membranes were from Nucleopore (Pleasanton, CA, USA). Total RNAs were extracted with the RNeasy mini kit from Qiagen (Valencia, CA, USA) and reverse-transcribed by Superscript III from Invitrogen (Carlsbad, CA, USA). LPS was purchased from Sigma–Aldrich (St. Louis, MO, USA). Primers for quantitative RT-PCR were obtained from Syntheza Inc. (Rehovot, Israel). All other reagents were of analytical grade.

2.2. Preparation of nanoparticles

Lipid-based nanoparticles were prepared as previously described, without surface modification [2,21]. Three types of NPs were prepared: neutral in charge (NPs) composed of HSPC and Chol at 4:1 mol ratio; negatively charged [(−)NPs] composed of HSPC:Chol:DSPG at 3:1:1 mol ratio; and positively charged [(+)NPs] composed of HSPC:Chol:DOTAP at 3:1:1 mol ratio. Briefly, multilamellar vesicles (MLV) were prepared by a lipid-film method and evaporated to dryness using a buchi-rotovap [2,21]. The lipid film was hydrated with Phosphate-buffered saline pH 7.4 to create MLV. Lipid mass was measured as previously described [2]. Resulting MLV were extruded into small unilamellar nano-scale vesicles (SUV) with a Thermobarrel Lipex extruder™ (Lipex biomembranes Inc., Vancouver, British Columbia, Canada) at 60 °C under nitrogen pressures of 300–550 psi. The extrusion was carried out in a stepwise manner using progressively decreasing pore-sized membranes (from 1, 0.8, 0.6, 0.4, 0.2, to 0.1 μm) (Nucleopore, Whatman), with 10 cycles per pore-size. NPs, (−)NPs and (+)NPs were stored at 4 °C until further use and not more than 2 weeks.

2.3. Entrapment of siRNAs in (+)NPs

siRNAs entrapment was done similar to the one reported in [6]. Briefly, the lipids were dissolved in chloroform:methanol (4:1, v/v). The organic solvent was evaporated under pressure at 60 °C for 30 min and the lipid film was flushed with N_2 gas to remove residual solvent. The lipid film was hydrated using a solution of siRNAs (Luciferase) with previously described sequence [2] in 5% dextrose (w/v) prepared using RNase-free dH_2O. Size reduction was performed as with conventional particles (see Section 2.2). siRNAs amounts that were entrapped were assayed by the RiboGreen assay (invitrogen) as previously described [2].

2.4. Particle size distribution and zeta potential measurements

Particle size distribution and mean diameter of NPs, (−)NPs, (+)NPs and siRNAs entrapped in (+)NPs were measured on a Malvern Zetasizer Nano ZS zeta potential and dynamic light scattering instrument (Malvern Instruments, Southborough, MA) using the automatic algorithm mode and analyzed with the PCS 1.32a. All measurements were done in 0.01 mol/l NaCl, pH 6.7, at room temperature.

2.5. Animal care and treatment

Animals (healthy C57BL/6 mice) were obtained from the animal-breeding center, Tel-Aviv University (Tel Aviv, Israel). Animals were maintained and treated according to National Institutes of Health guidelines. All animal protocols were approved by the Tel-Aviv Institutional Animal Care and Use Committee.

2.6. Blood biomarkers assays

Healthy C57BL/6 mice ($n = 5$/group) were given a single bolus intravenous (i.v.) injections of saline, or treated with NPs, (−)NPs or (+)NPs at a lipid concentration of 10 mg/kg body/mouse. 4 h or 24 h post initial injection, blood was drawn and the serum was obtained by centrifugation of the whole blood at 850*g* for 15 min. Liver enzyme levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) were determined by COBAS MIRA auto analyzer (Roche).

2.7. Bodyweight measurements

Healthy C57BL/6 mice ($n = 5$/group) were given bolus i.v. injections of saline, or treated with NPs, (−)NPs or (+)NPs twice weekly for 3 weeks (overall 6 doses/mouse of 10 mg/kg body/dose/mouse) and individual mouse bodyweight was monitored every 3 days using an Avery Berkel scale (Fairmont, MN, USA).

2.8. Splenocytes isolation and cell sorting

Healthy C57BL/6 mice were used for harvesting splenocytes. In some experiments C57BL/6 mice injected with saline, NPs, (−)NPs or (+)NPs (2 or 24 h post i.v. injection) were harvested. Isolation of splenocytes (1×10^8 splenocytes/mouse) from C57BL/6 wild-type (WT) or splenocytes from C57BL/10ScNJ-Tlr4*lps-del* ($TLR4^{-/-}$ mice) (a kind gift from Dr. G. Nussbaum, Hebrew University, Jerusalem, Israel) was preformed as previously described [2,22,23]. For cell sorting, 5×10^7 cells (range $1–10 \times 10^7$) were used. Cells were incubated with anti-mouse CD11b-Alexa700 (clone M1/70 – expressed on granulocytes, monocytes, macrophages, myeloid-derived dendritic cells, and natural killer cells), anti-mouse CD3ϵ-Alexa488 (clone: 145-2C11, T cells), and anti-mouse B220-PE-Cy5 (clone: RA3-6B2, B cells) all from BD Pharmingen (San Jose, CA, USA). Antibodies diluted in PBS and stained for 30 min at 4 °C. Rat IgG_{2b}-Alexa700, Armenian Hamster IgG1-Alex488, and Rat IgG_{2a}-PE-Cy5 diluted in PBS were used as matched isotype controls. Cells were washed with PBS twice and suspended in PBS- 2 mM EDTA. 7AAD was added to stain dead cells. Cell sorting was performed with a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA) equipped with blue, red, and violet lasers. Dead cells were excluded by 7AAD vs forward scatter (FSC) dot plots. Doublets were excluded from the cell population by side scatter (SSC)-W vs SSC-H and FSC-W vs FSC-H dot plots. Finally, bright $CD11b^+$, $CD3^+$ and $B220^+$ cells were selected and sorted using the cell sorter's purity option at a rate of 5000 events per second. Sorted populations were reanalyzed for purity and viability, and 1×10^6 cells of each population was collected into Eppendorff tubes and was treated with various NP formulations and then subjected to RNA extraction for further analysis of interferon (IFN) response analysis and cytokine induction. Alternatively, mice ($n = 5$/group) treated with saline, NPs, (−)NPs, and (+) NPs formulations as listed in Figs. 3 and 4, were collated into Eppendorff tubes with RNA extracting buffer using the RNeasy isolation kit from QIAGEN (Valencia, CA, USA) for IFN analysis and cytokine induction.

2.9. Interferon and cytokine induction assays

Mice splenocytes were isolated from the spleen as previously described [2,22,23]. Expression of interferon responsive genes (IRG) and cytokines were examined by quantitative RT-PCR. mRNA levels of IRG, as well as a panel of cytokines were quantified either after splenocytes were sorted for monocytes, T cells and B cells and mock-treated or treated with NPs, (−)NPs, (+)NPs and siRNAs entrapped in (+)NPs or upon intravenous administration of saline, NPs, (−)NPs or (+)NPs at 10 mg/kg body into C57BL/6 mice. LPS (100 ng/mL) was used as a positive control.

2.10. Quantitative RT-PCR

Quantitative RT-PCR using a Step one Plus Real time RT-PCR (ABI) was carried out as previously described [2]. RNA was extracted from the cells using RNeasy kit (Qiagen). Total RNA (1 μg) was reverse transcribed into cDNA using Superscript III from Invitrogen in a 25 μL reactions. GAPDH served as endogens control. Primers for mouse GAPDH, STAT1, OAS1, IFN-β, IL-2, TNF α, and IFN γ, were as previously described [2]. The following primer pairs were used:

IL-6 primers: forward primer: 5′-ATCCAGTTGCCTTCTTGGGACTGA-3′; reverse primer: 5′-TAAGCCTCCGACTTGTGAAGTGGT-3′.
IL-13 primers: forward primer: 5′-CTGTGAGCCTTGTCCTCCTC-3′; reverse primer: 5′-TTGGTGAGCCAGTGAGACG-3′.
IL-17 primers: forward primer: 5′-CATGCAGGAGGTGGTACCTT-3′; reverse primer: 5′-AGCTTCTTCTCGCTCAGACG-3′.
MX1 primers: forward primer: 5′-GAATAGCAACTCCATACCGTG-3′; reverse primer: 5′-GTATTAAAGGTTGCTGCTAATG-3′.
G1P2 primers: forward primer: 5′-GTGGTGCAGAACTGCATCTC-3′; reverse primer: 5′-GCCAGAACTGGTCTGCTTGT-3′.

2.11. Statistical analysis

In vitro data were analyzed using Student's *t*-test. Differences between treatment groups were evaluated by one-way ANOVA with significance determined by Bonferroni adjusted *t*-tests.

3. Results

3.1. Particle size distribution and surface charge measurements

To investigate whether surface charged NPs induce toxic effects and immune activation systemically, we prepared three types of NPs differing in their surface charge, namely anionic, cationic and

Table 1
Hydrodynamic diameter and zeta potential measurements of neutral, positively and negatively charged NPs.

NPs type	NPs lipid composition (mole ratio)	Hydrodynamic diameter (nm)	z potential (mV)
NPs	HSPC:Chol (4:1)	100 ± 18	−8.5 ± 2.4
(−)NPs	HSPC:Chol:DSPG (3:1:1)	104 ± 10	−59.2 ± 4.9
(+)NPs	HSPC:Chol:DOTAP (3:1:1)	107 ± 14	+54.3 ± 6.1
(+)NPs	HSPC:Chol:DOTAP: siRNAs (3:1:1:0.5)	223 ± 21	+38.1 ± 4.0

Each result is an average (±standard deviation) of 6 independent measurements. Particles were measured at pH 6.7, in ddH_2O with 10 mM NaCl, at 20 °C using a Malvern ZS Zetasizer.

neutral in charge NPs. All NPs were composed of HSPC and cholesterol, which form the basic lipid nanoparticle structure, while positively charged nanoparticles [(+)NPs] and negatively charged NPs [(−)NPs] were composed with the addition of DOTAP or DSPG, respectively. The quaternary ammonium head group in the DOTAP lipid contributed a positive charge to the NPs, while addition of DSPG with its orthophosphate head group donated a negative charge to the NPs. Dynamic light scattering (DLS) and surface charge (zeta potential) measurements were used to characterize and validate the NPs hydrodynamic diameter and charge of all three NPs types. As shown in Table 1, the average NPs size was in the range of 100 nm in diameter for all formulations with a narrow size distribution as evident by the small standard deviation values. siRNAs entrapped in the positively charged NPs increased their size to ~220 nm in diameter. NPs composed from HSPC and cholesterol had a zeta potential of −8.5 mV confirming their neutral charge at the measured pH. An addition of 20% mole of DSPG to the formulation changed the charge of the particles to negative (~−60 mV), while adding 20% mole DOTAP turn the particles into positively charged (+54 mV). Entrapment of siRNAs within (+)NPs neutralized some of the surface charge and reduced it to ~(+38 mV).

3.2. Positively charged NPs induce systemic toxicity in vivo

To examine the carriers' potential to induce systemic toxicity, we tested two global toxicity markers [2,24]: serum liver enzymes and body weight loss upon multiple injections.

High release of liver enzymes measured in the serum is considered an indication of hepatotoxicity [24]. Serum levels of the liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined in healthy C57BL/6 mice ($n = 5$/group) i.v. administrated with saline or with 10 mg/kg body NPs, (−)NPs or (+)NPs and at 2 time points (4 and 24 h post i.v. injection), blood was drawn to assess potential hepatotoxicity. This dose of 10 mg/kg body lipid NPs is at the low end of doses reported for lipid-based NPs [6,25]. While NPs and (−) NPs induced only mild changes in liver enzymes release that are considered well-tolerated [24] (Fig. 1A), administration of (+)NPs significantly enhanced liver enzymes release even after 4 h post injection and more robustly (3–6-fold higher than the mock-treated mice) 24 h post administration, indicating possible liver toxicity.

Changes in body weight are also considered a global toxicity marker [2]. To determine the changes in bodyweight, 8 weeks-old C57BL/6 healthy mice ($n = 5$/group) were i.v. injected with a dose of 10 mg/kg body NPs, (−)NPs, (+)NPs, or saline (mock-treatment), twice a week for 3 weeks. Changes in bodyweight were followed over a period of 27 days. NPs- and (−)NPs-treated mice did not decrease their bodyweight, however, their growth rate was smaller

A

	Mock		NPs		(-)NPs		(+)NPs	
Liver enzymes	4h	24h	4h	24h	4h	24h	4h	24h
ALT (U/L)	55 ± 7	62 ± 9	71 ± 15	108 ± 19	66 ± 8	97 ± 12	102 ± 21	**347 ± 31**
AST (U/L)	110 ± 17	127 ± 31	189 ± 37	174 ± 23	129 ± 18	156 ± 27	223 ± 21	**694 ± 54**
ALP (U/L)	100 ± 9	96 ± 8	123 ± 12	128 ± 21	97 ± 8	103 ± 10	156 ± 23	**276 ± 22**

B

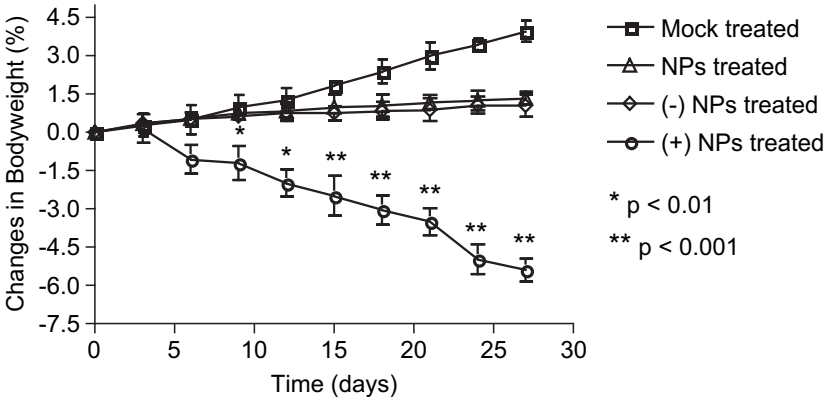

Fig. 1. Positively charged nanoparticles induce systemic toxicity in vivo. A. Serum levels of liver enzymes. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in C57BL/6 mice, 4 and 24 h post i.v. administration of 10 mg/kg body NPs, (−)NPs, (+)NPs or saline (mock). Values expressed in units per liter as mean ± SEM, ($n = 5$/group). B. Changes in bodyweight. C57BL/6 mice after i.v. injections of 10 mg/kg body NPs, (−)NPs, (+)NPs, or saline (mock) twice a week for 3 weeks. Values are expressed as mean ± SEM, ($n = 5$/group), $^{*}p < 0.01$; $^{**}p < 0.001$ vs mock-treated.

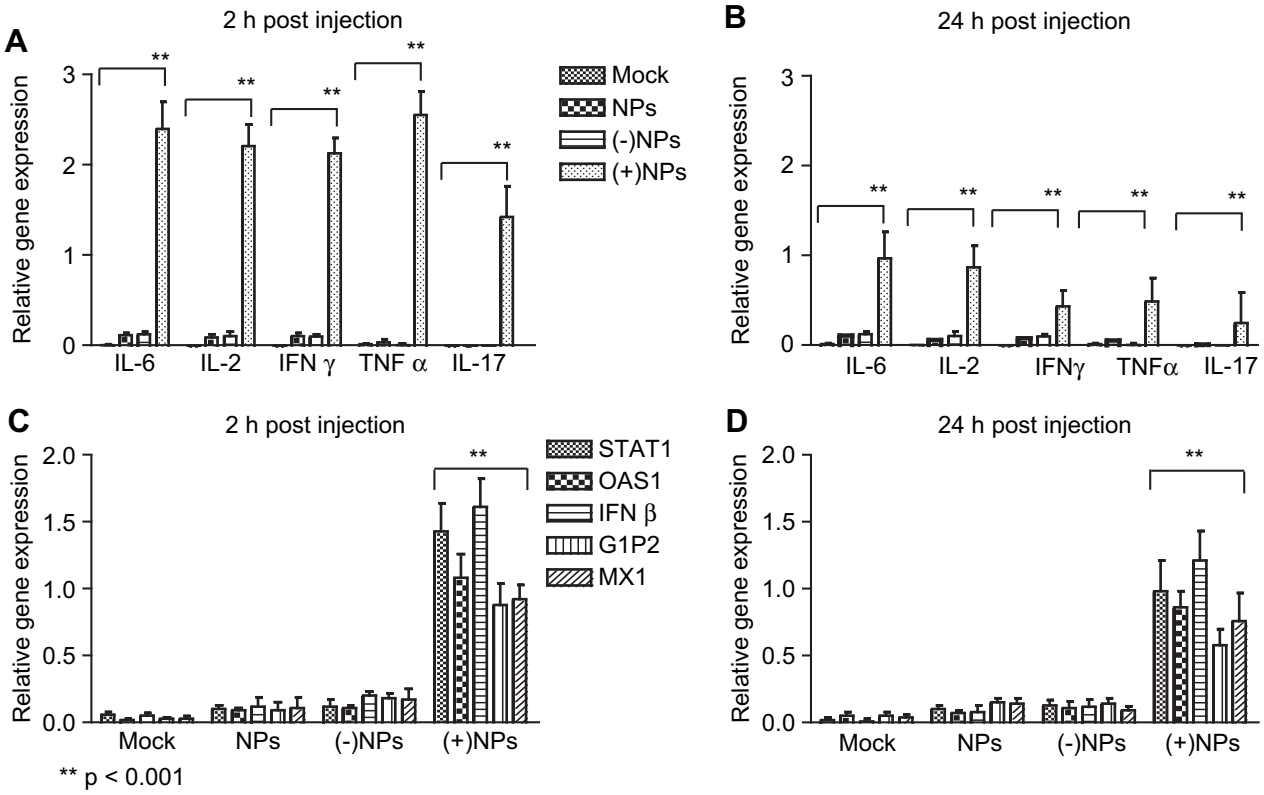

Fig. 2. Positively charged nanoparticles induce an inflammatory response in vivo. mRNA expression of cytokines and interferon responsive genes (IRG) in splenocytes harvested from mice treated with NPs, (−)NPs, (+)NPs, or saline (mock). Th1 cytokines mRNA expression level A. 2h post injection. B. 24h post injection. IRG mRNA expression level C. 2h post injection D. 24h post injection. Values are expressed as mean ± SEM normalized to GAPDH. ($n = 4$/group). $^{**}p < 0.001$ vs NPs and (−)NPs treatments.

than the mock-treated mice implying potential toxicity upon multiple injections (Fig. 1B). Mice treated with (+)NPs lost body-weight (~5.5% at day 27) confirming possible hepatotoxicity that correlates with the high serum liver enzyme levels.

3.3. Positively charged NPs induce pro-inflammatory response in vivo

Induction of pro-inflammatory cytokines and interferon responsive genes (IRG) can cause opposite trends such as hyper immune activation or global immune suppression (depending on various environmental conditions) and are usually not well defined when developing nanocarriers for various applications [2,10]. To determine whether intravenous administration of NPs induce pro-inflammatory cytokines and IRG expression, mice ($n = 4$/group) were mock-treated with saline or administrated with NPs, (−) NPs and (+)NPs. Splenocytes were harvested 2 and 24 h post systemic administration and mRNA of selected cytokines and IRG genes were measured using quantitative RT-PCR (qPCR). High induction in Th1 cytokines (IL-2, IFN γ, TNF α) and in Th17 cytokines (IL-17, and IL-6) was observed 2 h post i.v. injection of (+)NPs (Fig. 2A). This induction in expression was 10–24-fold higher than observed when NPs or (−)NPs were administered. There was almost no detectable induction of Th1 cytokines from the NPs and (−)NPs 2 h post i.v. injection, similar to mock-treated mice (Fig. 2A). A substantial decrease in cytokine induction (2.6–10-fold lower than expressed 2 h post administration) was observed 24 h post i.v. administration of (+)NPs (Fig. 2B) indicating a possible tolerance of the leukocytes to this stress. The induction of cytokines 24 h post administration of NPs and (−)NPs was as low as the mock-treated levels and on par with the cytokine induction observed 2 h post i.v. injection, suggesting that the basal level of cytokine induction was measured (Fig. 2B). Similar trend was observed when IRG expression was measured (Fig. 2C). High induction in IRG expression (15-fold higher than that of NPs or (−)NPs) was observed 2 h post i.v. injection of (+)NPs (Fig. 2C). 24 h post injection IRG expression was mildly reduced by 1.5–0.5-fold (Fig. 2D) suggesting a possible immune tolerance to the stress caused by the (+)NPs as seen in cytokine induction (Fig. 2A,B).

3.4. Positively charged NPs activate the immune response directly at the cellular level

Immune activation caused by delivery of (+)NPs may be mediated by their interaction with serum proteins, lipoproteins or extracellular matrix or by toxic signals released from an injured organ, such as the liver or spleen [26,27]. Alternatively, (+)NPs may interact directly with immune cells and lead to immune activation [26]. To examine this question, primary T cells, B cells and monocytes, were FACS sorted from healthy splenocytes and mock-treated or incubated with NPs, (−)NPs, and (+)NPs. 5 h post incubation, IRG and a panel of cytokines expression was measured using qPCR. LPS, is the primary component of the outer membrane of Gram-negative bacteria, which is an inducer of TLR4. Binding of LPS to TLR4 triggers various cell-signaling pathways, which lead to up-regulation of Th1 cytokines and interferon type I response [28,29]. Therefore, LPS was used in these experiments as a positive control. Correlated with the in-vivo experiments, induction of IRG expression was observed only with (+)NPs at 15–25-fold higher expression in all subtypes of leukocytes (monocytes ($CD11b^+$), T cells ($CD3^+$) and B cells ($B220^+$)) tested compared with NPs or (−)NPs treatment. The IRG expression levels when (+)NPs or LPS were used was similar (Fig. 3A). This IRG induction occurred without the presence of any potential mediator, thus immune response was elevated directly at

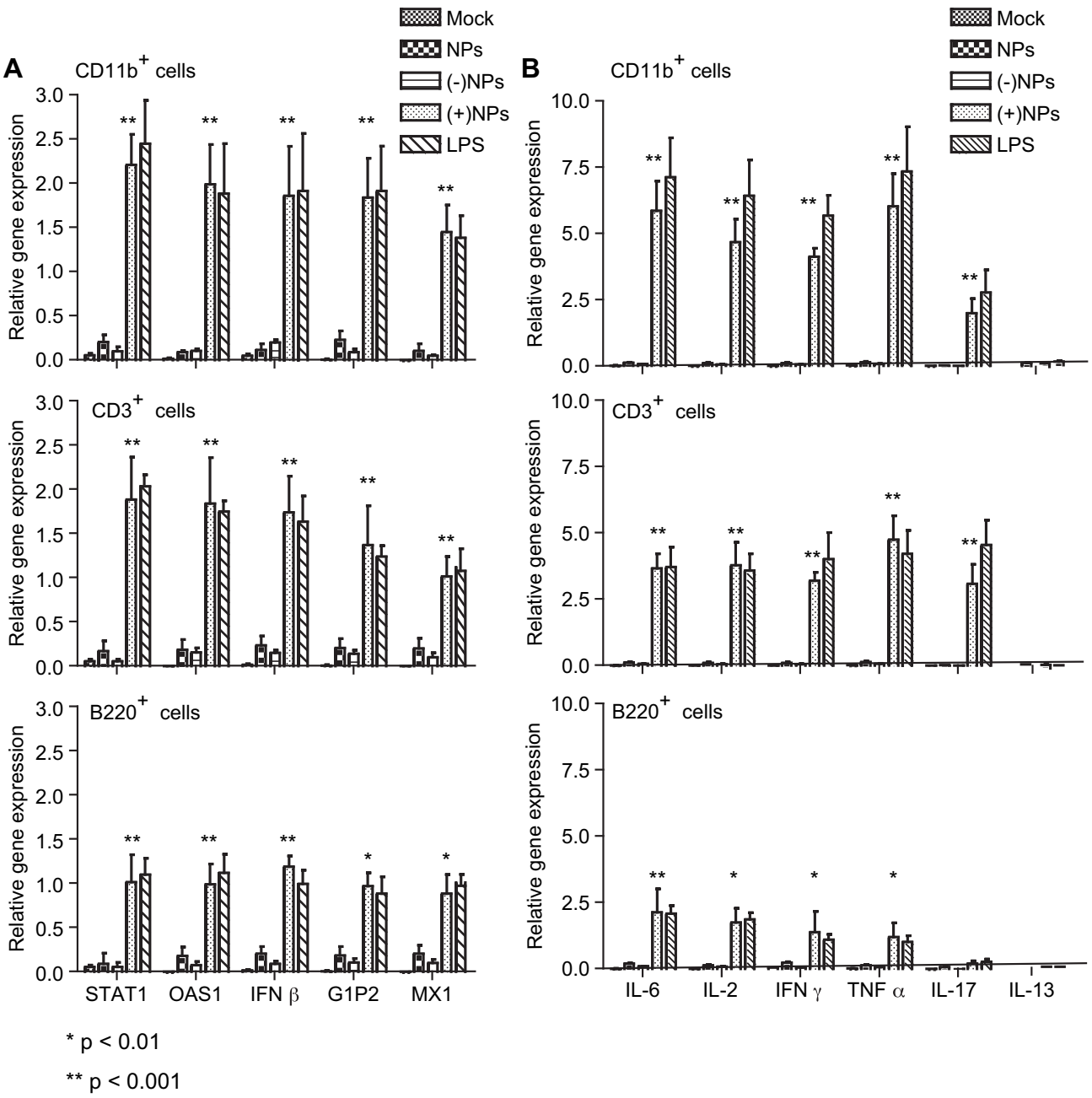

Fig. 3. Positively charged nanoparticles activate the pro-inflammatory response at the cellular level. mRNA expression of immune responsive genes in primary monocytes, ($CD11b^+$), T cells ($CD3^+$) and B cells ($B220^+$), isolated from WT splenocytes, measured 5h after treatment with NPs, (−)NPs, (+)NPs, saline (mock) or LPS .A. Interferon responsive gene mRNA levels normalized to GAPDH. B. Cytokines mRNA level normalized to GAPDH. Values expressed as mean ± SD. $^*p < 0.01$; $^{**}p < 0.001$ vs NPs and (−)NPs treatments.

the cellular level. Similar trend was observed when cytokines were examined (Fig. 3B). All leukocytes subpopulations induced Th1 cytokines (IL-2, IFN γ and TNF α) when treated with (+)NPs at 10–75-fold higher than treatment with NPs or (−)NPs at different levels (monocytes > T cells > B cells). Th1 induction was specific since no increasing in IL-13 expression, a representative Th2 cytokine, was observed (Fig. 3B). IL-17, a cytokine from the Th17 family has been shown to play a crucial part in the induction of autoimmune diseases [30,31]. Substantial expression of IL-17 mRNA levels in T cells and a mild expression in monocytes treated with (+)NPs (Fig. 3B) was observed. This may suppress some of the Th1 response as a balance between Th1 and Th17 as previously demonstrated in another setting [32].

3.5. Positively charged NPs activate the immune response through Toll-like receptor 4 (TLR4)

TLR4 activation induce both pro-inflammatory cytokines and type I interferon [28,33]. In addition, IL-13 is known to be expressed upon TLR2 activation but not by TLR4 activation [34]. This has raised the hypothesis that (+)NPs activate the TLR4 pathway. To test this hypothesis, primary $TLR4^{-/-}$ monocytes, T cells, and B cells were mock-treated or incubated with NPs, (−)NPs and (+)NPs. Five hours post incubation cytokines and IRG expression were measured using qPCR. The results demonstrate that (+)NPs operate in a TLR4 dependent manner since Th1 cytokines expression levels showed 60–75-fold decrease in

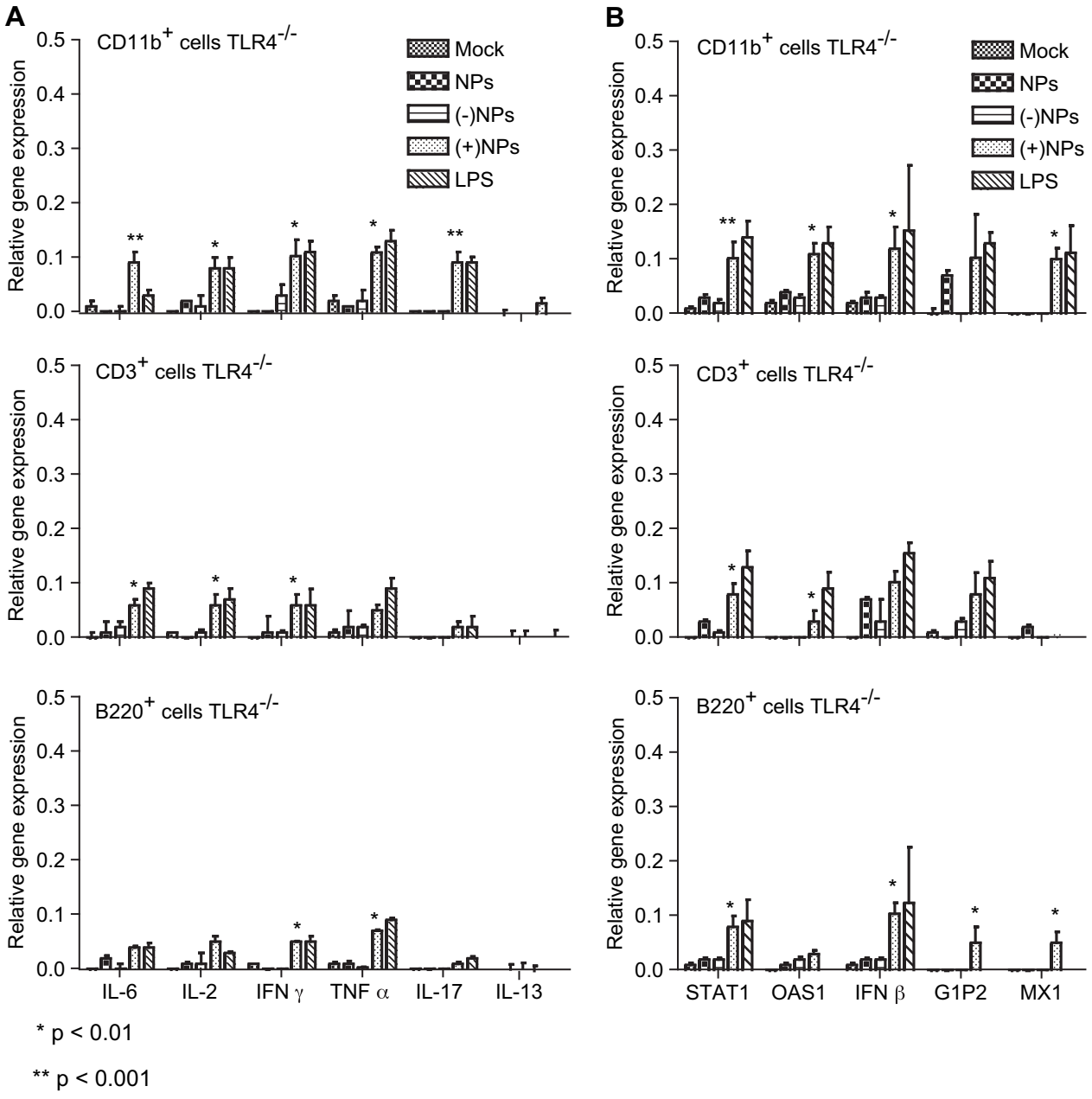

Fig. 4. Positively charged nanoparticles do not activate pro-inflammatory response in TLR4$^{-/-}$ leukocytes. mRNA expression of immune responsive genes in primary monocytes, (CD11b+), T cells (CD3+) and B cells (B220+) isolated from TLR4$^{-/-}$ splenocytes, measured 5h after treatment with NPs, (−)NPs, (+)NPs, saline (mock) or LPS. A. Cytokines mRNA level normalized to GAPDH. B. Interferon responsive gene mRNA levels normalized to GAPDH. *$p < 0.01$; **$p < 0.001$ vs NPs and (−)NPs treatments.

expression levels compared to WT leukocytes (Fig. 4A compare with Fig. 3B) and had a very low expression level on par with LPS (Fig. 4A). This pro-inflammatory response was dose dependent (see Supplemental Fig. 1). Treatment with NPs or (−)NPs had no change in cytokines expression levels when TL4$^{-/-}$ leukocytes were used, and was on par with the wt leukocytes levels (Fig. 4A compare with Fig. 3B). Similar trend was obtained when IRG expression levels were examined (Fig. 4B). 17–25-fold decrease in IRG expression levels were observed when (+)NPs were incubated with TL4$^{-/-}$ leukocytes compared with WT leukocytes (compare Fig. 4B with Fig. 3A) and was similar to the decrease in IRG expression when LPS was used (Fig. 4B). Treatment with NPs or (−) NPs did not induce any decrease in IRG expression, stringent the conclusion that (+)NPs activate TLR4.

3.6. Positively charged nanoparticles entrapping siRNAs activating a pro-inflammatory response in a TLR4-dependent manner

Entrapment of siRNAs within (+)NPs ((+)NPs-siRNAs) changes the physicochemical properties (see Table 1). To examine whether they are representative for applications involving siRNAs delivery, Pro-inflammatory cytokines and IRG of CD11b^{+} sorted cells were profiled in response to this formulation. These cells were used as model cells since they have shown the most profound induction of cytokines and IRG (Fig. 3).

These results showed that administration of (+)NPs-siRNAs induced immune response by upregulating Th1 cytokines and IRG. Compared with RNAi-free(+)NPs, there was a slight decrease in pro-inflammatory cytokines expression. This might be due to the

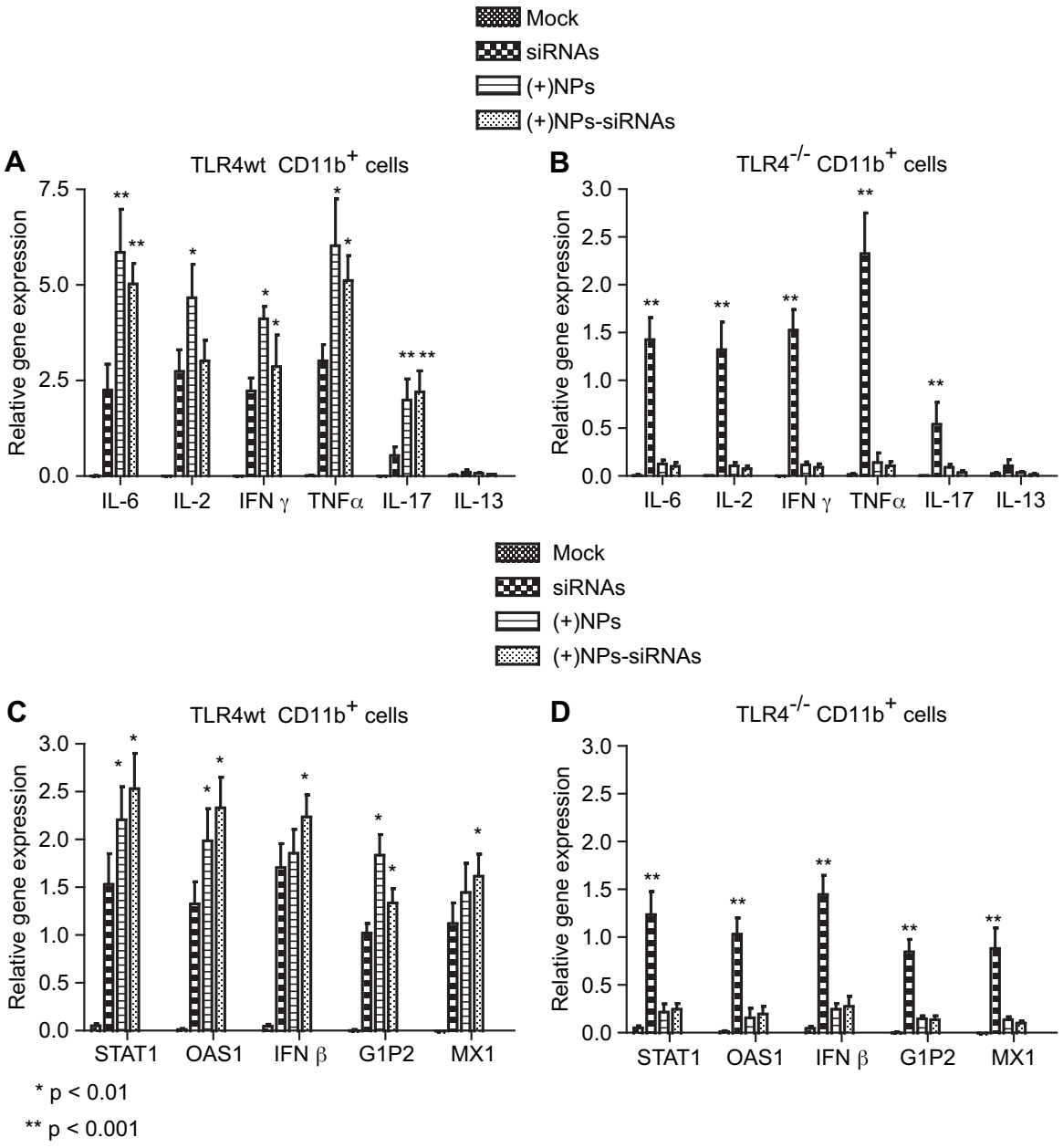

Fig. 5. Positively charged nanoparticles entrapping siRNAs activating a pro-inflammatory response in a TLR4 −dependent manner. Primary monocytes ($CD11b^+$) isolated from TLR4 wt and $TLR4^{-/-}$ splenocytes were mock-treated or treated with free siRNAs, (+)NPs and (+)NPs entrapping siRNAs (all at 10 μg/mL). mRNA of immune responsive genes was isolated 5 h posted treatment and quantified using qPCR. A. Cytokines mRNA levels normalized to GAPDH in $CD11b^+$ TLR4wt cells B. Same in $CD11b^+$ $TLR4^{-/-}$ cells. C. Interferon responsive gene mRNA levels normalized to GAPDH in $CD11b^+$ TLR4wt cells D. Same in $CD11b^+$ $TLR4^{-/-}$ cells. $^*p < 0.01$; $^{**}p < 0.001$ vs free siRNA.

entrapment of siRNAs in the (+)NPs which reduced the net charge of the particles from +54 mV to +38 mV (Table 1). However, no decrease in IRG expression was observed. This may point to different IRG induction pathways. Since the addition of siRNA is known to induce immune response [11–13], $CD11b^+$ sorted cells were incubated with free siRNAs as a positive control. A dramatic stimulation of Th1 cytokines and IRG was observed when free siRNAs, (+)NPs and (+)NPs-siRNAs were incubated with $CD11b^+$ cells (Fig. 5A and C). To test whether this induction was derived from the net positively charged NPs and not from the siRNAs, we incubated free siRNAs, (+)NPs and (+)NPs-siRNAs with $TLR4^{-/-}$ $CD11b^+$ sorted cells.

Immune induction was completely abolished in $TLR4^{-/-}$ cells treated with (+)NPs-siRNAs, while a profound effect of cytokine and IRG induction was still observed in cells treated with free siRNAs (Fig. 5B and D).

4. Discussion

Here we have shown that systemic administration of positively charged lipid nanoparticles ((+)NPs) in vivo is toxic as showed by several global indicators. Administration of (+)NPs to mice induced hepatotoxicity (as evident by high levels of serum liver enzymes) as well as weight lose of 5.5% upon multiple injections. Moreover, at the cellular level, (+)NPs treatment stimulated inflammatory response by elevating both Th1 and Th17 cytokines and IRG similar to treatment with LPS.

Our experimental data, although correlated with other in-vivo study showing inflammatory response in intratracheal administration [35], is not in agreement with the *in vitro* toxicity study by Tanaka et al. that showed DOTAP liposomes up-regulate co-stimulatory molecules CD80 and CD86 but not pro-inflammatory

cytokines in bone marrow dendritic cells [36]. This contradiction may suggest that intravenous administration uses a mediator component not existing in cell culture model. However, based on our study, (+)NPs stimulation is done directly at the cellular level as we showed for B cells, T cells and monocytes (including dendritic cells). Another explanation for the inconsistency may rely on NPs composition. In our system, (+)NPs are composed of DOTAP, cholesterol and phosphatidylcholine (PC), which are required for NPs stability and for improvement of cellular uptake [37,38] and not solely on DOTAP. It has been shown that an addition of PC as a co-lipid to cationic liposomes effect inflammatory response as an adjuvant [39].

In the present study we show that the induction of Th1 cytokines by (+)NPs is specific since no increase in the Th2 cytokine, IL-13, is observed. This result suggests that the immune activation might be via TLR4, since IL-13, unlike other cytokines, is specifically induced by other TLRs activation and not TLR4 [34].

In addition, it has been shown that cationic liposomes administration reduced cell viability by elevating reactive oxygen species (ROS) generation [40,41]. ROS elevation was shown to play an important role in LPS-induced pro-inflammatory cytokines production [42,43]. Furthermore, cells stimulation with LPS-induced ROS production and NF-κB activation in a TLR4 dependent manner [44]. Taking into account these results, it was reasonable to assume that (+)NPs induce toxicity through TLR4. Indeed by using TLR4 knockout mice, we showed that (+)NPs immune activation is mainly TLR4 dependent, since (+)NPs did not stimulate inflammatory response similar to the one that was observed by LPS. Moreover, we showed that while both free siRNA and siRNAs entrapped in (+)NPs induced immune response in $CD11b^{+}$ cells, only free siRNA was able to induce pro-inflammatory response in $TLR4^{-/-}$ $CD11b^{+}$. This provides a strong support for TLR4 dependency in (+)NPs-siRNAs toxicity and strengthens the understanding that (+)NPs-siRNAs effect on pro-inflammatory response is not due to the siRNA component, which elevates immune response in a TLR4-independent manner, as suggested by previous studies [11–13], but derived by the cationic component of (+) NPs.

The understanding of DOTAP lipid-based NPs effects on leukocytes is crucial for RNAi delivery strategies in general and specifically in their application in cancer and inflammation [45]. Cationic NPs are being used in cancer therapy studies [46–48] and the relation between inflammation, innate immunity and cancer is widely investigated [49–51]. The involvement of the TLR family in general and TLR4 specifically in cancer is actively studied [52,53]. The activation of TLR4 by LPS was shown to induce proliferation, activation of NF-κB pathway and chemo-resistance in ovarian cancer [29] as well as elevation of migration and invasion in both colon cancer cells and pancreatic cancer cells [23]. These results must be taken into account when considering applying (+)NPs in cancer therapy. Activation of TLR4 by (+)NPs entrapping siRNAs may lead to cancer promotion and progression as well as increase angiogenesis. Therefore, other strategies that do not relay on cationic components should be devised. Those will probably utilize mechanisms of receptor-mediated endocytosis for internalization of the RNAi payloads into specific cell types.

5. Conclusions

Here we investigated the toxic effect of positively charged lipid nanoparticles in vivo. Under certain experimental conditions, a formulation of DOTAP-based nanoparticles has induced systemic toxicity, Th1 cytokines expression and activated a type I interferon response. We found that these cationic nanoparticles activate toll-like receptor 4 expressed on leukocytes in a specific manner. This study lays the foundation for evaluating different types of nanoparticles and their immune toxicity that may impact the development of cationic nanocarriers as delivery systems for nucleic acids in general and RNAi in particular.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biomaterials.2010.05.027.

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