Choose your models wisely: How different murine bone marrow-derived dendritic cell protocols influence the success of nanoparticulate vaccines *in vitro*

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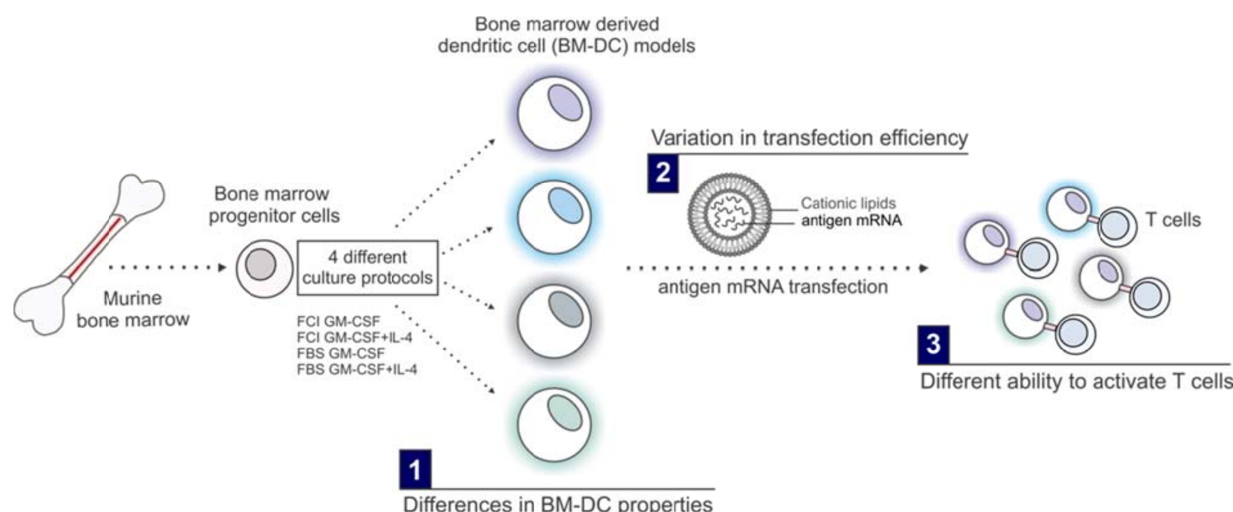
ABSTRACT

Dendritic cell (DC)-based cancer vaccination has shown great potential in cancer immunotherapy. As a result, novel nanoparticles aiming to load DCs with tumor antigens are being developed and evaluated *in vitro*. For this, murine bone marrow-derived DCs (BM-DCs) are most commonly used as model DCs. However, many different protocols exist to generate these cells. Therefore, we investigated to what extent different BM-DC culture protocols impact on the immunobiology of the cells, as well as their response to particulate antigens. We evaluated 4 different BM-DC protocols with 2 main variables: bovine serum and cytokine combinations. Our results show distinct differences in yield, phenotypical maturation status and the production of immune stimulatory and immune suppressive cytokines by the different BM-DCs. Importantly, we demonstrate that the antigen-loading of these different BM-DCs via transfection with mRNA lipoplexes results in large differences in transfection efficiency as well as in the capacity of mRNA-transfected BM-DCs to stimulate antigen-specific T cells. Thus, it is clear that the BM-DC model can have significant confounding effects on the evaluation of novel nanoparticulate vaccines. To take this into account when testing novel particulate antigen-delivery systems in BM-DC models, we propose to (1) perform a thorough immunological characterization of the BM-DCs and to (2) not only judge a particle's potential in cancer vaccination based on transfection efficiency, but to include an evaluation of functional end-points such as T cell activation.

Keywords: dendritic cell, mRNA, cancer vaccination, nanoparticle

Chemical compounds studied in this article: DOTAP (PubChem CID: 6437371); DOPE (PubChem CID: 9546757); CholEsteryl BODIPY® FL C12 (PubChem CID 70682631); SIINFEKL (PubChem CID: 71311993)

Graphical abstract:



1. INTRODUCTION

Since the first discovery of dendritic cells (DCs) as a novel cell type in mice by noble prize-winner Ralph M. Steinman, DCs came to be known as nature's adjuvant [1]. This title was awarded to them due to their unique capacity to elicit antigen-specific immune responses. DCs continuously sample their environment, engulfing foreign material and presenting it on their surface to naïve T cells. Importantly, DCs can provide the necessary co-stimulatory signals to activate T cells and promote their differentiation into cytotoxic T lymphocytes (CTLs) and helper T cells (Th) in case of $CD8^+$ and $CD4^+$ T cells respectively. The more the DC's role in initiating immunity was unraveled, the more its potential use in medicine came to light. For instance, by modifying DCs to present tumor-associated antigens (TAAs) or HIV-antigens, specific antitumor or anti-HIV immune responses can be evoked [2-4]. Research in this field of therapeutic DC vaccines flourished and has resulted in the FDA-approval of the first DC-based vaccine in 2010 (Provenge®, marketed by Dendreon) [5, 6].

In search of novel strategies for effective antigen-loading of DCs to combat cancer or viral infections, experiments are generally performed on *in vitro* generated DCs. The main reason for this is the low number of *in vivo* DCs that can be isolated from different tissues [7-9]. As a result, different protocols were established for the *in vitro* production of DCs, generated from murine bone marrow precursors or from the peripheral blood monocytes found in human blood [10-13]. By culturing these murine and human monocytic precursors for a number of days in the presence of appropriate cytokines, their differentiation into murine bone marrow-derived DCs (BM-DCs), and human monocyte-derived DCs (MoDCs) will be induced. This way convenient DC models for *in vivo* studies of the DC functionality, phenotype, immunogenic potential and antigen-presentation capacity were established. Importantly, these models gained in popularity among researchers who are developing novel particulate systems for the delivery of protein or nucleic acid antigens to DCs. However, it should be noted that although these methods result in the generation of large numbers of cells with DC-like properties, they do not seem to correspond to any of the DC subsets that under normal circumstances populate the mouse lymph nodes. Instead, they rather, but not completely, resemble "emergency" or "inflammatory" DCs that only occur in inflamed lymph nodes [14-16]. Therefore, *in vitro* generated monocyte-derived DCs need to be considered as simplified models for a complex *in vivo* situation, and conclusions from experiments using these cells should be drawn with the necessary caution. An additional critical factor that complicates the use of DCs *in vitro*, is the plethora of protocols that are available for the generation of these cells. The differentiation of bone marrow into BM-DCs can be achieved with either GM-CSF (Granulocyte-macrophage colony-stimulating factor) alone or in combination with IL-4 (interleukin-4), but also other factors, including Flt3L (FMS-related tyrosine kinase 3 ligand) or IL-3 have been employed at different concentration ratios [14, 17, 18]. Changing the cocktail and concentrations of cytokines can result in major differences in DC phenotype. In addition, other factors such as cell culture medium, the timeframe for DC generation from bone marrow precursors, additional purification steps, mouse age and especially growth factors that are present in the sera that are routinely added to the culture medium may decide the type of BM-DCs that are produced. Obviously, these differences make it difficult to compare results obtained in different *in vitro* models. For one, the variability of BM-DCs generated via different protocols has already lead to substantial debate on an immunological level. For instance, Lutz et al. observed major differences in the immune stimulating properties of

BM-DCs based on the serum and cytokine cocktails used during their differentiation from bone marrow cells [19, 20].

Importantly, this variability in BM-DC protocols can also be observed in research on novel antigen-delivery systems. In recent years, numerous reports on the design of novel biomaterial systems to deliver antigenic material to DCs, packaged in nano- and microparticles have been published. These delivery vehicles are becoming increasingly complex, often packaging antigen-coding nucleic acid sequences (plasmid DNA and mRNA) instead of proteins or peptide antigens and aiming for controlled or triggered antigen-delivery to antigen-presenting cells [21-23]. In the initial evaluation of these particles, BM-DCs are often used as model DCs. And here as well, a vast diversity in the BM-DC generation protocols is observed. We hypothesize that these changes in the culture protocols for BM-DCs *in vitro* have important repercussions for their response to particulate antigens, and that a careful choice of the BM-DC protocol is warranted in order to give newly designed delivery systems a full chance of success.

In this study, we aim to investigate the effect of using four differently generated model DCs on their immunological properties, as well as their response to particulate antigen. More specifically, we chose to use lipoplexes containing antigen-coding mRNA. mRNA is particularly interesting for the delivery of antigens to DCs, as it offers several advantages: (a) by introducing antigen mRNA, the antigenic protein will end up in the DC cytoplasm and will therefore preferentially be presented in MHC-I which results in de induction of $CD8^+$ T cells; (b) by introducing the nucleic acid sequence encoding an entire protein, immune responses against multiple epitopes can be induced; (c) mRNA is translated in the cytoplasm and therefore does not need to cross the nuclear membrane, in contrast to plasmid DNA and (d) mRNA is not considered as a gene therapeutic, as it does not encompass the risk of genomic integration [24]. For these reasons, we used particles consisting of mRNA complexed to cationic lipids, and evaluated their use in different BM-DC models.

2. Materials and Methods

2.1. Dendritic cell culture

Primary murine bone marrow-derived DC (BM-DC) cultures were generated from C57BL/6 mice. Female C57BL/6 mice were purchased from Harlan Laboratories and housed in an SPF facility according to the regulations of the Belgian law and the local Ethical Committee. Mice were euthanized and bone marrow was flushed from the hind limbs. The red blood cells in the resulting single cell suspension were lysed (Pharm Lyse Buffer, BD Biosciences, Erenbodegem, Belgium) and the collected cells were seeded in 100 mm Not TC-Treated polystyrene Culture Dishes (Corning®, Amsterdam, The Netherlands) at $2x10^6$ cells ml^{-1} in 15 ml. The cell culture medium used was RPMI 1640 (Gibco-Invitrogen, Merelbeke, Belgium) supplemented with penicillin/streptomycin/L-glutamine (1%, Gibco-Invitrogen) and β-mercaptoethanol (50 µM, Gibco-Invitrogen) and 5% serum. Two different types of serum were used: Fetal Bovine Serum (FBS 5%, Batch n°RSE30013, HyClone™, Pierce, Rockford, IL, USA) and FetalClone™ I (FCI 5%, Batch n°AXD36551, HyClone™). To promote differentiation of the monocytes into BM-DCs, cytokines were added: GM-CSF alone (20 ng ml^{-1}, Peprotech, Rock Hill, NJ) or a combination of GM-CSF (10 ng ml^{-1}) with IL-4 (10 ng ml^{-1}, Peprotech). On day 3 of the culture,

an additional 15 ml complete cytokine-supplemented culture medium containing GM-CSF (40 ng ml^{-1}) or GM-CSF and IL-4 (both at 20 ng ml^{-1}) was added. On day 5, all cells were collected by centrifugation (5 min at 300 g), resuspended in the appropriate culture medium at 10^6 cells ml^{-1} and seeded in 24 well plates for experiments ($5x10^5$ cells per well). For every experiment, 1 single batch of bone marrow cells was used to generate the 4 different BM-DC cultures in order to exclude bias due to animal-related effects.

2.3. Cell yield, viability and purity

At day 5, when the cells were collected from the petri dishes, cell yield was determined by counting the collected cells with trypan blue exclusion of dead cells (Sigma-Aldrich, Bornem, Belgium). DC purity and cell viability were evaluated the next day, via anti-CD11c-allophycocyanin (APC) surface staining (eBiosciences, Vienna, Austria) and a SYTOX® green nucleic acid stain (Molecular Probes/Invitrogen, Merelbeke, Belgium), respectively. The cells were collected from the wells, washed in FACS buffer (phosphate buffered saline (PBS, Gibco-invitrogen), supplemented with 5% bovine serum albumin, BSA (Sigma-Aldrich, Bornem, Belgium)) and incubated with a staining buffer containing both the antibody and 45 nM SYTOX® green for 30 min at 4°C. After additional washing steps, the cells were analyzed by flow cytometry using a FACSCalibur and CellQuest Pro software (BD Biosciences).

2.4. Microscopy

Transmission microscopy images of the cells obtained via the different BM-DC generation protocols were recorded using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium), equipped with a Plan APO 40x DIC water immersion objective lens (Nikon) and suitable optical elements to obtain differential interference contrast (DIC) transmission images. For this, the cells were seeded in 35 mm MatTek glass bottom culture dishes (MatTek Corporation, MA, USA) on day 5 of the culture, and imaged on day 6.

2.5. BM-DC phenotype analysis

The effect of different sera or cytokine combinations on the DC phenotype was investigated by examining the expression of the maturation markers CD40, CD86 and MHC-II on the DC surface. For this, the cells (at day 6 of the culture) were either untreated, or supplemented with E.coli derived lipopolysaccharide (1 µg ml^{-1} LPS, Sigma-Aldrich) to induce maturation. 24 h after LPS addition, the cells were collected, washed with flow buffer and surface stained for the DC marker CD11c-APC in combination with staining for either CD40-phycoerythrin (PE), CD86-PE or MHC-II-fluorescein isothiocyanate (FITC) (all BD Biosciences) for 30 min at 4°C. After additional washing steps, the cells were analyzed by flow cytometry.

2.6. mRNA

Luciferase, eGFP, and ovalbumin (OVA) mRNA were produced by *in vitro* transcription from pBlue-Luc-A50, pGEM4Z-GFP-64A and pGEM-Ii80tOVA plasmids [25]. The plasmids were purified using a QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands) and linearized using Dra I (for the pBlue plasmid) or Spe I (for the pGEM plasmids) restriction enzymes (Promega, Leiden, The Netherlands). Linearized plasmids were used as templates for the *in vitro*

transcription reaction using the T7 mMessage mMachine kit (Ambion, Life Technologies, Ghent, Belgium). The resulting capped and polyadenylated mRNAs were purified by DNase I digestion, LiCl precipitation and washing with 70% ethanol. The mRNA concentration was determined by measuring the absorbance at 260 nm. mRNAs were stored in small aliquots at -80°C at a concentration of 1 µg/µl.

2.7. mRNA lipoplexes

Cationic liposomes, containing 50% DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and 50% DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) (both Avanti Polar Lipids, Alabaster, AL) were prepared by transferring the appropriate amounts of lipids, dissolved in chloroform into a round-bottom flask. To prepare fluorescent mRNA lipoplexes, 1 mol% CholEsteryl-BODIPY® FL C12 (Molecular Probes/Invitrogen) was added. The chloroform was evaporated under nitrogen and the resulting lipid film was rehydrated in RNase-free water (Ambion) to obtain a final lipid concentration of 1 mg/ml. The resulting DOTAP/DOPE liposomes were sonicated for 15min in a bath sonicator (Branson Ultrasonics, Dansbury, USA), after which they were mixed with mRNA to obtain mRNA lipoplexes at a cationic lipid-to-mRNA charge (N/P) ratio of 10 in OptiMem® (Gibco-Invitrogen). The produced liposomes and lipoplexes were subjected to a size and zeta potential quality control prior to use, using a Malvern Zetasizer nano-ZS (Malvern Instruments Ltd, Worcestershire, UK).

2.8. mRNA lipoplex loading and transfection of DCs

Uptake and transfection efficiency of mRNA lipoplexes were evaluated in the four differently generated BM-DCs. Lipoplex loading was performed on BM-DCs at day 6 in 24 well plates. The cell culture medium was removed and the mRNA lipoplexes, dispersed in OptiMem® were added for 2 h at 37°C (1 µg mRNA per well, N/P=10). For uptake experiments, fluorescent mRNA lipoplexes containing luciferase mRNA were used. After the 2 h incubation period, the cells were collected, washed with FACS buffer and extracellular fluorescence was quenched using trypan blue (1:1 diluted in FACS buffer for 5 min at RT). Then, the cells were washed, surface stained for CD11c-APC and the uptake of fluorescent mRNA lipoplexes was evaluated by flow cytometry. The transfection efficiency of the lipoplexes was evaluated using eGFP mRNA, and after the 2 h incubation of the BM-DCs with the particles in OptiMem®, the cells were re-cultured in the appropriate cell culture media. After 24 h incubation at 37°C, the eGFP-transfected cells were collected, surface stained for CD11c-APC and expression of eGFP was analyzed by flow cytometry. Untreated cells served as a negative control.

2.9. *In vitro* T cell activation assay

In order to assess the potential of the BM-DCs to prime antigen-specific $CD8^+$ T cells, an *in vitro* OT-I activation assay was performed. In this assay, OVA lipoplex transfected DCs were co-cultured with OT-I cells, which have a transgenic T cell receptor that recognizes the MHC-I restricted OVA-peptide SIINFEKL. Untreated and eGFP-transfected DCs served as negative controls. As a positive control, DCs loaded with SIINFEKL peptide (1 µg ml^{-1}, Eurogentec, Seraing, Belgium) were used. 5 h after transfection, the cells were matured for 2 h with LPS. Then, the DCs were collected, washed, and seeded per 10^4 DCs in a U-bottom 96 well plate (Falcon, BD Biosciences), for co-incubation with 10^5 OT-I cells (derived from the spleens of OT-

I transgenic mice, Charles River). After 5 days, the cells were collected, surface stained for CD8-APC (BD Pharmingen) and the T cell activation marker CD25-PE (MACS Miltenyi, Leiden, The Netherlands) and analyzed by flow cytometry.

2.10. ELISA

Supernatants of untreated and LPS-stimulated DCs were screened for the presence of IL-10 and IL12p70. Supernatants of DC-T-cell co-cultures were assayed for IFNγ and IL-2. Cytokine concentrations were measured via ELISA (all Ready-SET-Go!® ELISA kits, eBioscience) according to the manufacturer's instructions.

2.11. Statistical analysis

All data are presented as mean ± standard deviation. Presented data are representative for at least 3 independent experiments performed on cells derived from the bone marrow of different donor mice, except for the uptake experiments. Statistical analyses were performed using a One-Way ANOVA with Bonferroni correction.

3. RESULTS

3.1. DC yield, purity and viability

We tested a limited number of variations that are commonly encountered in murine BM-DC generation protocols. Based on previous results, we chose to use BM-DCs at day 6 of the culture as this, in our hands, results in the largest cell yield and the lowest percentages of mature DCs in untreated samples. A first variable we tested, is the cytokine combination that is used to induce differentiation into BM-DCs. Initially, co-supplementation with GM-CSF and IL-4 was used, whereas more recent studies are performed on cells that were generated with GM-CSF alone. In order to investigate the influence of IL-4, we generated BM-DCs with cytokine supplementation of GM-CSF in the presence or absence of IL-4. A second important parameter is the serum that is added to the BM-DC culture medium as a source of nutritional and growth factors. In general, using different batches of fetal bovine serum (FBS) is known to cause variation in cell viability and growth rates due to large batch-to-batch variation in the levels of both defined and undefined growth factors present. Besides batch differences, varying types of serum have been used and tested for BM-DC generation. Therefore, we chose to use two different types of sera within the cell culture medium: regular FBS and FetalClone™ I serum (FCI). According to the manufacturers' information, the latter is supplemented with additional growth factors, but contains lower levels of certain immunosuppressive growth factors such as transforming growth factor beta-1 (TGF-β1). By changing these two parameters, we obtained four different protocols for the generation of BM-DCs and first investigated the influence of these parameters on cell yield, cell viability and DC purity.

A first observation after 6 days of culturing murine bone marrow cells in the four different culture media, was the difference in cell number. When the growth media are supplemented with FCI, expansion of the cell number is observed, with a 80% increase in cell count when GM-CSF is used alone, and 40% increase when a combination of GM-CSF and IL-4 is used. In case of FBS-supplementation, on the other hand, the cell number decreases after 6 days of culture,

resulting in a 40% and even 60% reduction in cell count when cultured when GM-CSF alone or combined with IL-4, respectively.

In all cases, the resulting cell population is quite heterogeneous, as can be seen in transmission microscopy images in **Figure 1A**. These show that in all 4 BM-DC cultures, the cells can grow both adherent to the petri dish surface, as well as suspended within the culture medium. In addition, the size and shape of the cells can largely vary. In all populations, a fraction of the cells display a DC-like appearance, with dendrites protruding from the cell membranes. To evaluate the viability of the cells after 6 days of culture, a SYTOX® green nucleic acid stain was performed. As shown in **Figure 1B**, although there is a trend towards lower cell viability when IL-4 is added to the medium, the differences observed were not significant, and for all protocols tested, the percentage of viable cells exceeded 80%.

In order to determine exactly how many BM-DCs can be obtained from each of the 4 culture circumstances, the cells were surface-stained for CD11c, a well-known DC marker, and analyzed by flow cytometry. The results in **Figure 1C** and **1D** indeed support the microscopic ascertainments, indicating that a portion of the cells in the population expresses CD11c and can therefore be identified as BM-DCs. A percentage of CD11c positive cells over 80% can be reached when cells are cultured in medium supplemented with FBS and GM-CSF alone, whereas the DC purity in the other cultures is limited to 50%. When these purity results are combined with the differences in total cell yield at day 6 of the culture, FCI-supplemented medium will provide the largest number of DCs, and GM-CSF alone is superior over a combination of GM-CSF and IL-4.

Often, researchers selectively collect either the suspension or the adherent fraction to use in their experiments. In our hands, $CD11c^+$ BM-DCs were found at high percentages in both suspension and adherent fractions, therefore, we chose to collect and use all cells for further experiments.

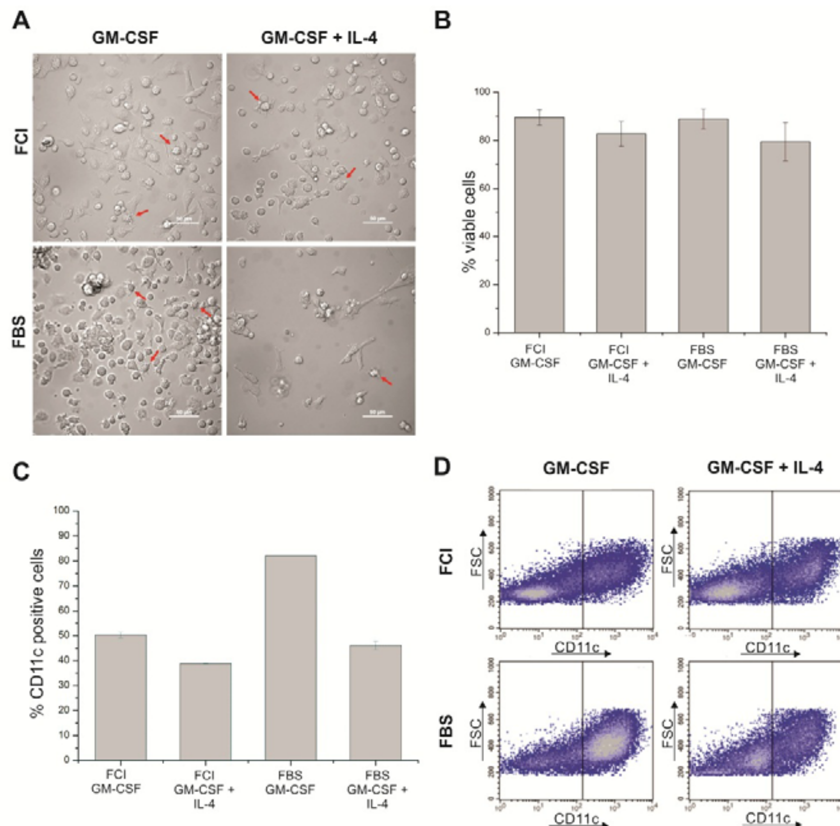


Figure 1: Morphology, viability and purity of different BM-DC cultures. Images show (A) Transmission microscopy images of BM-DCs generated via the 4 different protocols on day 6 of the culture. Red arrows indicate cells with a DC-like appearance; (B) Graphic representation of cell viability measured via SYTOX® green nucleic acid staining (n=3); (C) Graph showing the purity of the BM-DC cultures based on staining for CD11c (n=6), with representative scatterplots shown in (D). Results were analyzed with a one-way ANOVA with Bonferroni correction. There were no significant differences between the BM-DC cultures with respect to cell viability (B). Regarding the DC purity (C), the observed differences were all statistically significant ($p < 0.001$).

3.2. DC phenotype

An important phenotypical parameter that can provide information on the immunological properties of BM-DCs, is their maturation status. During maturation, DCs shift in function from antigen-uptake to antigen-presentation, a process that is accompanied by increased expression of different molecules that are required for effective antigen-presentation. As their presence is a crucial prerequisite for DCs to become effective T cell activators, we evaluated to what extent well-known maturation markers CD40, CD86 and MHC-II are present on the surface of the BM-DCs generated via the different protocols. For this, both untreated (blank) and BM-DCs where maturation was induced by co-incubating them with bacteria-derived lipopolysaccharde (LPS) were surface-stained for both the DC marker CD11c and the different maturation markers.

The results of flow cytometric analysis can be found in **Figure 2**. In case of the blank DCs, the same trends could be observed for all maturation markers tested. First of all, BM-DCs generated in presence of IL-4 exhibit a more mature phenotype, which demonstrate that IL-4 enhances the maturation process. Secondly, changing the serum in the cell culture media, also has a significant effect on the phenotype of the generated BM-DCs. In general, supplementation with FBS results in increased maturation marker expression compared to FCI. For effective activation of effector cytotoxic T lymphocytes by DCs, a fully mature DC phenotype is crucial. Even more so, numerous researchers have reported that antigen presentation by immature or partially mature DCs will induce antigen-specific tolerance rather than immunity [26]. Therefore, we studied the phenotypical properties of the different BM-DC cultures after overnight incubation with LPS, a known toll-like receptor 4 (TLR4) ligand, which is expected to induce complete maturation. Indeed, in all of the DC populations, there is a marked increase in the maturation marker expression level. However, these maximal levels vary gravely. Especially in case of BM-DCs cultured with FCI and GM-CSF alone, the percentage of fully mature CD86 and MHC-II expressing cells is limited to 40%, whereas percentages over 90% can be reached when FBS is used. For CD40 expression, differences between the different LPS-stimulated BM-DCs are less pronounced.

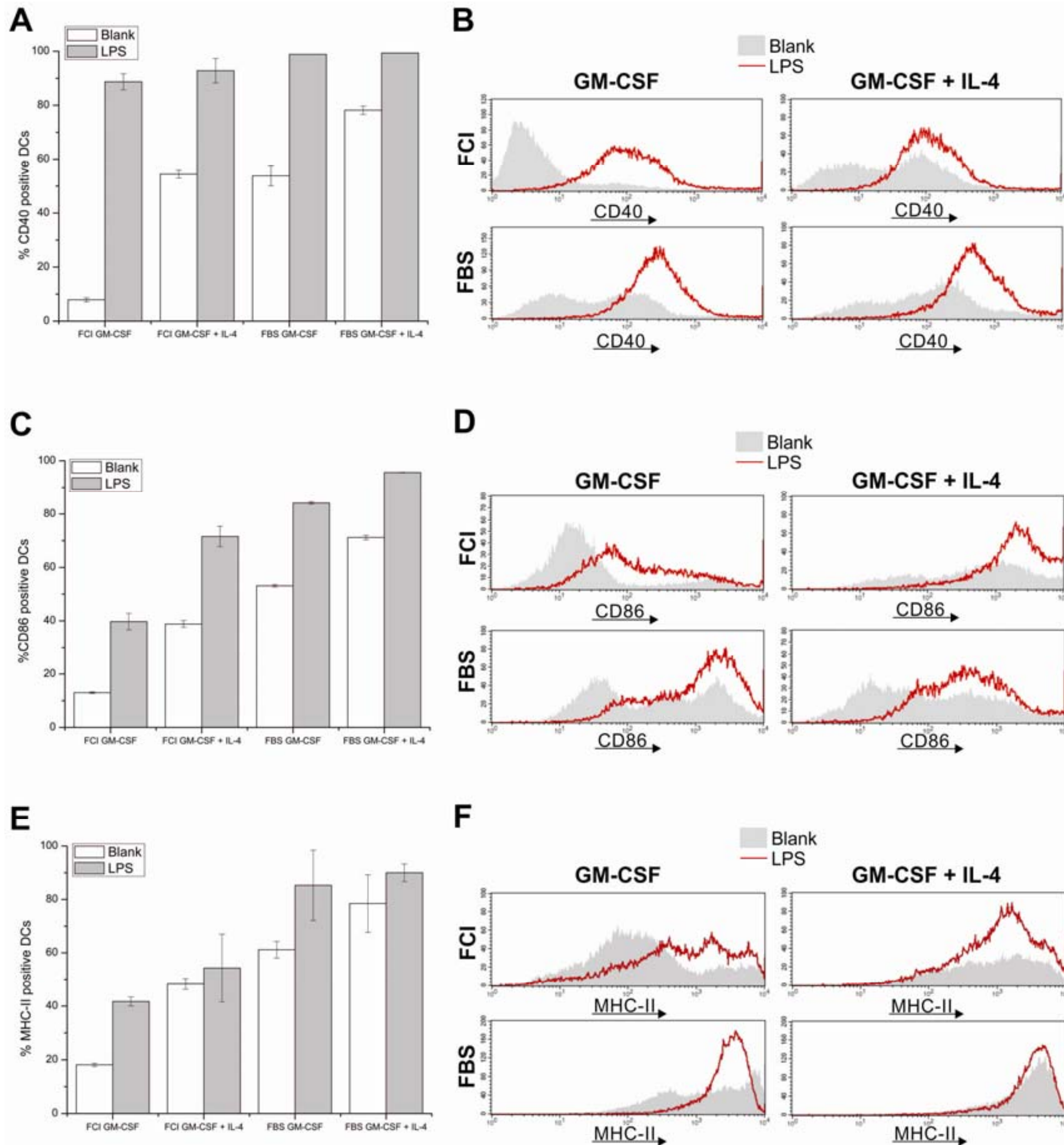


Figure 2. Expression of maturation markers by blank and LPS-stimulated DCs. Untreated (blank) and LPS-stimulated DCs (LPS) were analyzed for the expression of different maturation markers. DCs were gated based on CD11c staining. Images show (A) percentages of CD40 expressing DCs with (B) representative histograms; (C) percentages of CD86 expressing DCs with (D) representative histograms and (E) percentages of MHC-II expressing DCs with (F) representative histograms. (*n*=3)

Besides expression of molecules needed for antigen presentation and T cell activation on the DC surface, the cells also produce a number of cytokines that can skew T cell responses towards immunity or tolerance. We evaluated the production of two different cytokines by means of ELISA assays: the inflammatory cytokine IL-12p70, which is crucial for the activity of effector T cells and natural killer cells and on the other hand, the immune suppressive cytokine IL-10. As shown in **Figure 3A**, the production of IL-12p70 corresponds nicely to the maturation marker

expression by the cells. IL-4 addition as well as utilization of FBS for the culture medium will enhance the IL-12p70 production. The production of IL-10, which is a mediator of immune tolerance, also points towards a preferable situation when FBS and a combination of GM-CSF and IL-4 are used, as these both lead to a significant reduction in IL-10 production (**Figure 3B**).

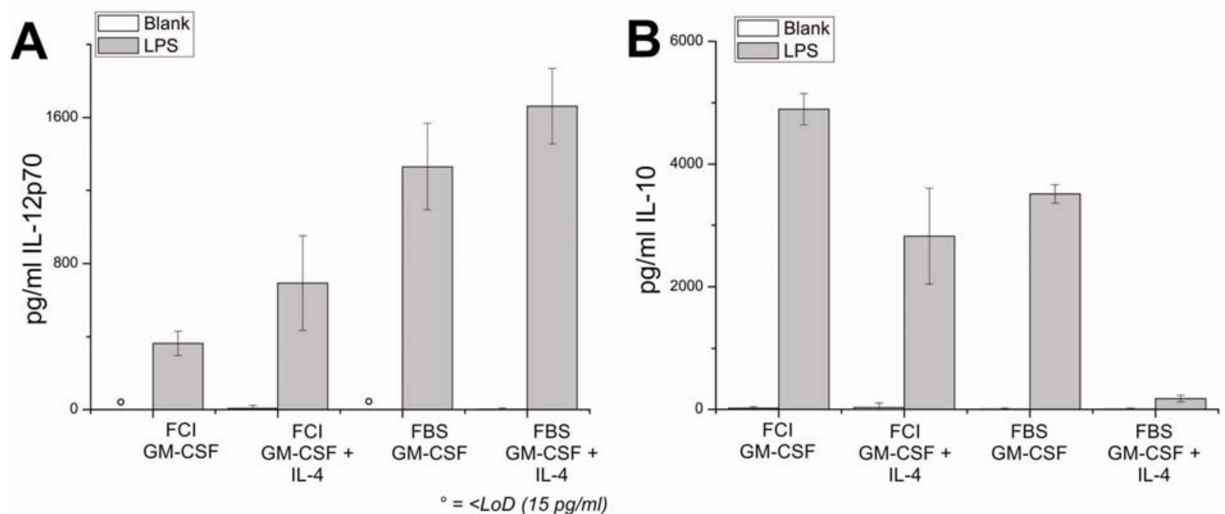


Figure 3. Cytokine expression by untreated and LPS-stimulated DCs. DC culture supernatants were collected after 24h of incubation in blank or LPS-supplemented culture medium. The supernatants were assayed for the immunostimulatory cytokine IL-12p70 (A) as well as the immune suppressive IL-10 (B). Graphs are summaries of 2 independent experiments (*n*=6).

3.3. mRNA lipoplex uptake and transfection potential

The previous experiments have exposed large differences in BM-DC yield, phenotype and cytokine production, when the cells are cultured via different protocols. Importantly, all of these protocols have been used to generate BM-DCs for the evaluation of novel antigen-delivery systems designed for potential use in DC-based vaccination. Therefore, we wanted to investigate to what extent the choice of BM-DC model could influence the results obtained with particulate antigens. As a model antigen-delivery system, we chose mRNA lipoplexes, since mRNA is becoming increasingly investigated as a source of antigen, and this requires more of the DCs' own machinery for translation and presentation of the antigens, compared to the use of protein- or peptide-antigens [27]. For the evaluation of such nucleic acid vaccines *in vitro*, transfection efficiency is employed as one of the main parameters and this can make or break the chance for further success. Therefore, we investigated if changes in the BM-DCs culture protocols influence the uptake and transfection potential of mRNA lipoplexes.

We chose to prepare DOTAP/DOPE-lipoplexes with a cationic lipid-to-mRNA charge (N/P) ratio of 10. At this N/P ratio, complete complexation of mRNA is observed. This was supported by zeta potential measurements, which exhibited a drop in surface charge, from 64± 6 mV for the cationic liposomes to 51 ± 7 mV after mRNA complexation to the lipids. mRNA complexation

resulted in an ~ 1.5 fold increase in mean particle size, as determined by DLS measurements. Mean particle sizes for liposomes and lipoplexes was 177 nm and 274 nm with a polydispersity index of 0.3 for both particles.

Overall, DCs exhibiting a more mature phenotype are expected to be less efficient in endocytosis and phagocytosis. This can be related to their function: mature DCs focus on antigen presentation, rather than antigen uptake [28]. To evaluate to what extent this affects mRNA lipoplex uptake by the cells, we incubated the different BM-DC cultures with fluorescently labeled mRNA lipoplexes. After 2 h of incubation, we evaluated the internalization of the mRNA lipoplexes by means of flow cytometry. We observed efficient uptake of the nanoparticles by the cells: in all BM-DC models tested, the percentage of lipoplex-loaded CD11c-positive cells was over 96%. However, when looking at the amount of intracellular mRNA lipoplexes, we could observe large differences in the mean fluorescence intensity (MFI) between the different BM-DC models, as shown in **Figure 4A**. As could be expected, the extent of nanoparticle uptake nicely correlated to the phenotypical maturation of the cells: more mature cells were indeed less efficient in engulfing the fluorescent mRNA lipoplexes.

In order to evaluate and to compare the efficiency of mRNA lipoplexes to transfect the different generated BM-DCs, mRNA encoding enhanced green fluorescent protein (eGFP) was used. eGFP mRNA lipoplexes were added to the cells as described in the materials and methods section. After overnight incubation, DCs were surface-stained for CD11c and levels of eGFP expression were evaluated by flow cytometry. The results of the flow cytometry analysis are presented in **Figure 4B-C**. Each DC culture was significantly transfected, but transfection efficiencies differed greatly from one culture to another, ranging from minimally 10% and maximally 37%. Clearly the choice of the BM-DC generation protocol highly influences the transfection efficiency of mRNA lipoplexes. Interestingly, for both sera tested the addition of IL-4 resulted in a significant reduction in transfection. Furthermore, this was more profound in the FBS-supplemented culture, which would suggest an additional influence of FBS-supplemented medium compared to FCI-containing medium. When GM-CSF was used alone, no significant difference in transfection efficiency was observed between the DCs cultured in FBS or FCI.

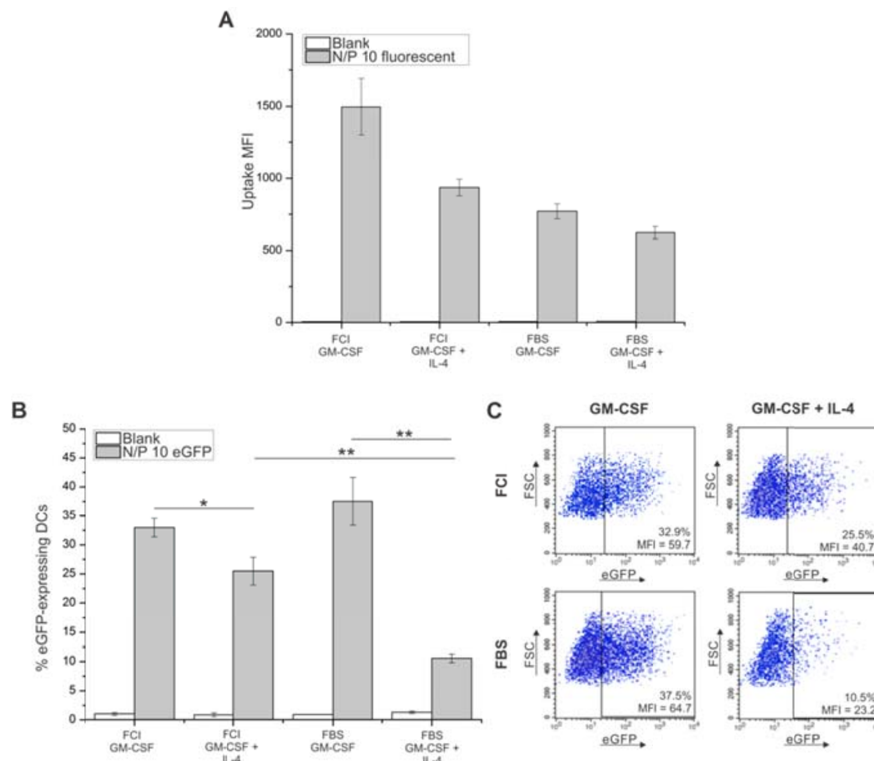


Figure 4. Uptake and transfection efficiency of mRNA lipoplexes. (A) MFI of BM-DCs after 2 h incubation with fluorescently labeled mRNA lipoplexes. Untreated cells served as blanks (*n*=6). (B) percentages of eGFP transfected DCs 24h after incubation with mRNA lipoplexes. Representative flow cytometry scatterplots are shown in (C). DCs were gated based on CD11c surface staining. Data represent mean ± SD and analysis was performed by one-way ANOVA with Bonferonni correction. (n=3, *$p < 0.05$, **$p < 0.01$).

3.4. *In vitro* T-cell activation

The key feature of DCs is their capacity to activate antigen-specific T cells, and to induce their differentiation into effector T cells. Since cancer immunotherapy mainly focuses on the induction of CTL responses directed against the tumor cells, we wished to evaluate the potential of different BM-DCs, loaded with mRNA lipoplexes, to induce antigen-specific $CD8^+$ T cell activation. For this, we performed a OT-I T cell activation assay. OT-I cells carry a transgenic CD8 T cell receptor which specifically recognizes the MHC-I restricted OVA peptide SIINFEKL. Therefore, if these OT-I cells are stimulated by mature DCs that present SIINFEKL in the context of MHC-I, these cells will be activated. This activation is accompanied by an increased expression of CD25 on the T cell surface.

As demonstrated in **Figure 5A**, all BM-DCs that were loaded with SIINFEKL peptide (positive control) were capable of inducing expression of CD25 in over 90% of OT-I cells. Changing cytokines or serum merely has a slight influence on the percentage of CD25 positive OT-I cells after 5 days of co-culture. When antigens were loaded into the DCs via mRNA lipoplexes, CD25 expression is comparable to the SIINFEKL positive control, indicating extensive antigen presentation in MHC-I by the mRNA-transfected BM-DCs. Only in the BM-DCs cultured with both GM-CSF and IL-4 in FBS-containing medium, mRNA-transfection results in a lower CD25

expression on OT-I T cells. The differences between the different BM-DC protocols are more prominent when the amount of CD25 expressed by the cells is studied, rather than the percentage of CD25-positive cells. For this, we looked at the MFI of the CD25-PE stain on the OT-I cells. The results in **Figure 5B** demonstrate that a higher CD25 expression could be detected in co-cultures of OT-I cells and OVA transfected BM-DCs either generated with FCI and GM-CSF + IL-4 or generated with FBS and GM-CSF alone. The lowest CD25 expression was observed with FBS and GM-CSF + IL-4 supplemented BM-DCs (Figure 5B).

Upon activation, the T cells can start proliferating, which often coincides with the production of IL-2. Moreover, if $CD8^+$ T cell activation leads to differentiation into CTLs, the cells will start secreting IFNγ, a type II interferon that enhances the CTL activity. Therefore, we measured the secretion of IL-2 and IFNγ in the DC-T cell co-culture supernatant via ELISA. The IL-2 production can be partly linked with the expression of CD25, pointing towards superior T cell activation by BM-DCs either cultured with GM-CSF and IL-4 in FCI-supplemented medium or with GM-CSF alone in FBS-supplemented medium. Importantly, the most remarkable differences were observed in the IFNγ production. Overall, BM-DCs cultured in FCI containing medium could double the IFNγ secretion by OT-I T cells compared to BM-DCs cultured with FBS. A more detailed statistical analysis of the results in this section can be found in supplementary data (Figure S1).

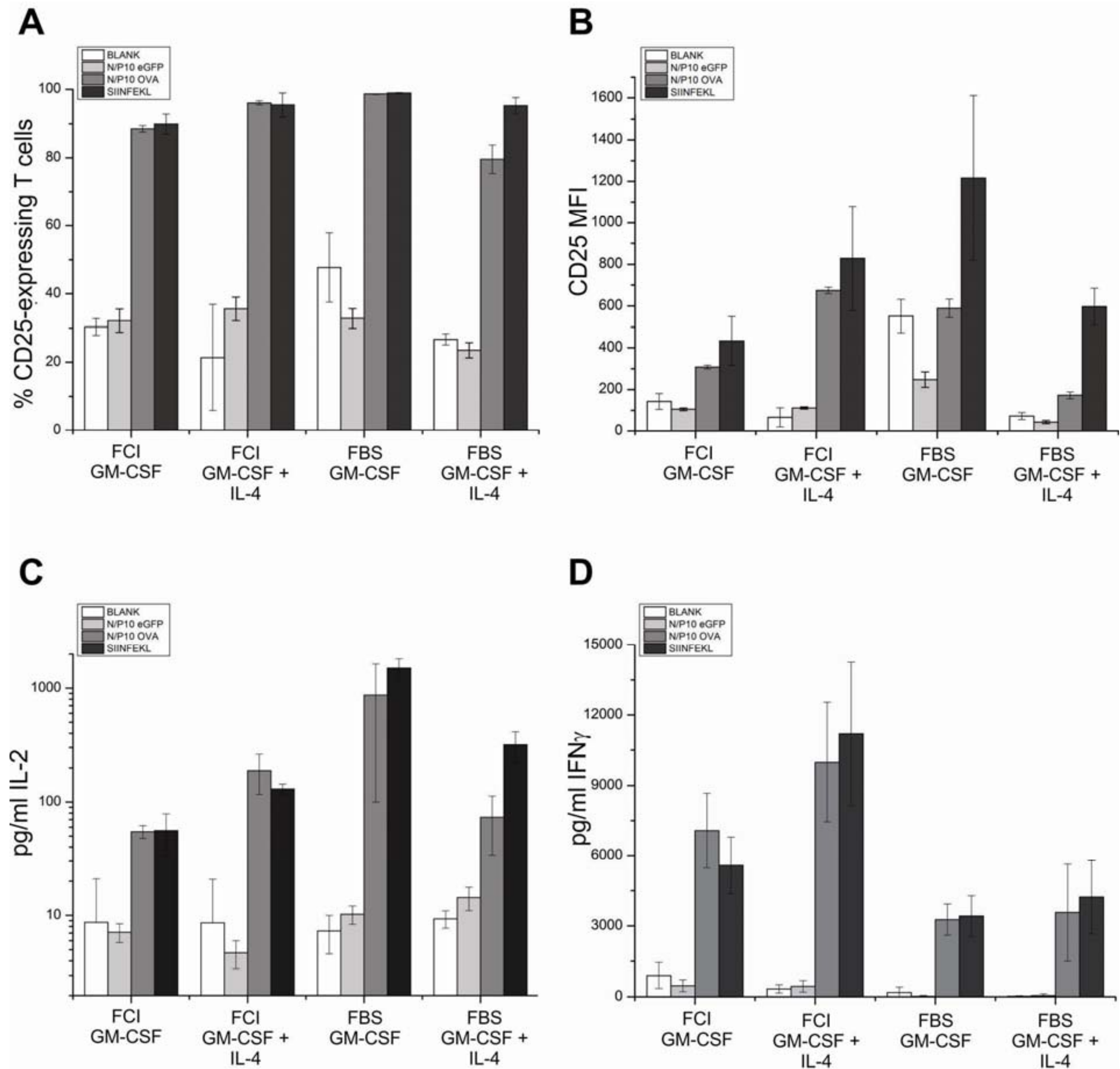


Figure 5. Antigen-specific T cell activation by mRNA lipoplex transfected DCs. OT-I T cells were co-cultured with DCs tansfected with eGFP or OVA mRNA. Blank and and SIINFEKL-loaded DCs served as negative and positive controls respectively. After 5 days, T cell activation was evaluated by measurement of CD25 expression and cytokine measurements. Figures show (A) % of CD25 positive OT-I T cells with (B) corresponding CD25 mean fluorescence intensity (MFI); (C) IL-2 production and (D) IFNγ production. *n*=3 for all experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni correction (represented in Supplementary Data, Figure S1).

4. DISCUSSION

BM-DCs are extensively used as *in vitro* model systems to investigate DC immunobiology, as well as for the evaluation of novel particulate vaccines. Due to large variation in protocols to generate BM-DCs from bone marrow precursors [29, 30], we hypothesized that the validation of novel antigen-delivery nanoparticles might be influenced on the BM-DC culture that is used for their evaluation. Several studies reported a number of crucial factors that can impact the yield, purity and phenotype of BM-DCs generated [19, 31]. Based on the work of Lutz et al., main variables are (a) mouse properties, (b) growth factors and (c) serum supplementation [19]. To exclude the first variable, we opted to consistently use female C57BL6 mice at 7 weeks of age. With regards to the two other variables, we screened recent literature on novel nanoparticulate vaccines to find out which growth factor combinations and sera are most widely used. Most researchers evaluate novel nanoparticles in BM-DCs grown in RPMI medium supplemented with GM-CSF alone (20 ng/ml) or with IL-4 (10 ng/ml) in addition to GM-CSF (10 ng/ml). As for the serum, Lutz et al. described this to be most important factor influencing the type of BM-DCs generated [19]. Therefore we chose to work with two different types of bovine serum: FBS and FCI.

After 6 days of culturing red blood cell-depleted murine bone marrow cells in the different cell culture media, the percentage of $CD11c^+$ DCs ranges from 39 to 80%. The highest BM-DC purity can be reached when FBS serum and GM-CSF alone are used. In accordance with previous reports, addition of IL-4 to the medium reduces the yield and purity of the BM-DCs [31]. It should be noted that other factors, such as the duration of the BM-DC culture, or the implementation of additional, and often quite complex, purification steps, can result in cultures with larger percentages of $CD11c^+$ cells [10, 29, 32]. Often, cells growing adherent to the culture dishes are excluded in order to increase BM-DC purity. In our hands, however, for all culture media tested, the adherent cell fraction contained a substantial portion of CD11c-expressing BM-DCs. This confirms previous results obtained by Li et al., who showed that there is no reason to discard adherent cells in BM-DC cultures as junk cells [33]. Overall, practically all BM-DC protocols will finally result in a cell population containing a (large) fraction of $CD11c^+$ BM-DCs as well as a fraction of non-DCs. These two subpopulations are expected to behave differently with regards to e.g. endocytotic capacity, maturation marker expression and cytokine production. Therefore, in order to assess the impact of novel nano- and micromaterials on DCs *in vitro*, it is important to selectively investigate the response of $CD11c^+$ cells, rather than the total cell population. Assays where no further selection or gating of CD11c-expressing cells is possible (e.g. cell counts, many cytotoxicity assays, DC-T cell co-cultures, other assays performed on the total population), can be impacted by these large variations in BM-DC purity. This should be taken into account in the design and set-up of new experiments.

Substantial differences in surface marker expression can also exist within the CD11c-positive population. This was made obvious by performing antibody staining against maturation markers CD40, CD86 and MHC-II, where we demonstrated the presence of both immature as well as mature BM-DCs in all cultures. Importantly, addition of IL-4 increases the proportion of mature DCs. This can be explained by the induction of "spontaneous" maturation of generated BM-DCs, which is known to be enhanced by the presence of IL-4 [29, 34, 35]. In addition, replacing FBS by FCI induces a significant reduction of maturation marker expression by the BM-DCs. In a study of Lutz et al., high serum levels of the liver enzymes glutamic oxaloacetic transaminase

(GOT) and lactate dehydrogenase (LDH) have been correlated with high percentages of mature cells [19]. For the batches of FBS and FCI we used, this correlation was not observed: the FCI used contained high levels of both liver enzymes, whereas merely a small fraction of the DCs exhibited a mature phenotype. Even though the effect of the serum compound is prominent, due to the complexity of these bovine products, we could not pinpoint the exact cause of the maturation-differences.

In nanoparticle research, a first step towards success is the efficient uptake of the nanomaterials by the target cells. In case of DCs, their internalization capacity is closely related to their maturation status: although mature DCs are still capable of performing receptor-mediated endocytosis, aspecific endocytosis as well as phagocytosis and macropinocytosis are markedly reduced in mature DCs compared to their immature counterparts [28]. This was also evidenced by our mRNA lipoplex uptake experiment, showing less extensive mRNA lipoplex uptake by BM-DCs that exhibit a more mature phenotype. Therefore, ideally, antigen-containing nanoparticles would be evaluated in immature BM-DCs. However, a striking observation was the reduced maturation capacity of the more immature BM-DC subsets. For example, BM-DCs cultured with FCI and GM-CSF alone, exhibit the lowest portion of mature DCs in untreated samples, but upon TLR-4 ligation maximally 40% of the BM-DCs will up-regulate CD86 and MHC-II. In contrast, well over 80% of BM-DCs generated in the presence of FBS medium, will up-regulate these markers upon LPS stimulation. This reduced responsiveness to maturation stimuli is nicely confirmed by cytokine measurements, showing that these specific BM-DCs also produce low levels of immune stimulatory IL-12p70 and high levels of the immune suppressive IL-10. Such phenotypical properties are expected to impact the immune responses that can be induced by these specific DCs. In addition, it is important to consider that for DC vaccines to be effective, efficient antigen-presentation by fully mature DCs is required. Since different reports have made clear that co-formulation of antigens and maturation stimuli will result in superior immune responses, the purpose of nanoparticulate vaccines has shifted from mere antigen delivery, to dual-modality particles that should simultaneously load DCs with antigens, and induce maturation of DCs [36, 37]. Therefore, immune stimulant such as the TLR ligands CpG and poly(I:C) have been incorporated into nanoparticulate vaccines [38]. As these dual-modality particles aim to induce complete maturation, it would undoubtedly be disadvantageous to evaluate them in a maturation-resistant BM-DC model. Therefore, a careful choice and characterization of the BM-DC model for the intended purpose is required.

In addition to immunological properties, we wished to assess to what extent novel nanoparticulate vaccines can lead to different results when tested in one BM-DC culture versus another. For this, we chose to work with mRNA lipoplexes, since mRNA is an increasingly investigated source of antigen in novel DC vaccines [27]. When DCs are loaded with nucleic acid sequences encoding tumor antigens, the transfection efficiency is used as a critical measure for the amount of antigen delivered, and hence the main criterion to evaluate new nanoparticulate vaccines. An evaluation of the transfection efficiency of mRNA lipoplexes in our 4 different models, revealed large differences, ranging from 10% to 37% mRNA expressing BM-DCs, even though the exact same nanoparticles were used. If transfection efficiency is the main parameter, it is now clear that the model BM-DC used for transfection experiments is a confounding factor. Do we then use the right means of scoring novel transfection-based particulate vaccines? First of all, it should, at all times, be kept in mind that the final end-point of the vaccine is to elicit antigen-specific T cell responses. And this is probably not a question of numbers. As reviewed by Gilboa

et al., high efficiency transfection of DCs with antigen does not necessarily correlate with improved immune stimulatory effects [39]. In addition, it was previously suggested that no analytical method can compare to the sensitivity of T cells to detect presented antigens [40, 41]. Therefore, as a concluding experiment, we evaluated to which extent the observed transfection efficiencies could lead to activation of antigen-specific $CD8^+$ T cells *in vitro*. After co-culturing naïve OVA-specific $CD8^+$ T cells with mRNA lipoplex-loaded BM-DCs, significant antigen-specific T cell activation could be observed in all models. However, the differences observed in the extent of T cell activation point towards influences of both phenotypical parameters as well as variations in transfection efficiency. For instance, BM-DCs grown in FCI and GM-CSF-supplemented medium showed low expression of molecules involved in antigen presentation (MHC-II) and T cell activation (CD86 and IL-12). As a result, even after efficient loading with antigen-mRNA, merely low T cell stimulation could be detected. A similar reasoning is valid for phenotypically superior BM-DCs (e.g. BM-DCs resulting from culture with FBS in the presence of IL-4), where a markedly low transfection efficiency is the likely cause for the deficient activation of T cells.

To conclude, *in vitro* data obtained with novel nanoparticles are not only the result of the nanoparticle's capacity to load DCs with antigenic material. Although BM-DCs are routinely used as a model for *in vivo* DCs, they should always be considered as simplified models for a complex *in vivo* situation. By screening a limited number of parameters, we were able to demonstrate extensive differences in BM-DC yield and immunological properties. Moreover, we showed that the BM-DC model in which novel nanoparticles are tested, acts as an important confounding factor in both transfection efficiency and T cell activation assays. This not only makes it hard to evaluate the potential of novel nanoparticles for DC vaccination purposes, but also renders it difficult to compare results obtained in different research groups using different BM-DC models. We wish to stress that our aim was not to identify an optimal BM-DC protocol, but to expose the bias of the model on the functional outcome of the nanoparticle. Therefore, we propose to: (1) include a thorough immunological characterization of the BM-DCs model used; (2) in addition to transfection efficiency, always evaluate functional end-points, such as T cell activation. Only if these two criteria are met, can the impact of the model's confounding effect be assessed, and can the true value of a novel particulate vaccine be revealed.

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SUPPLEMENTARY DATA

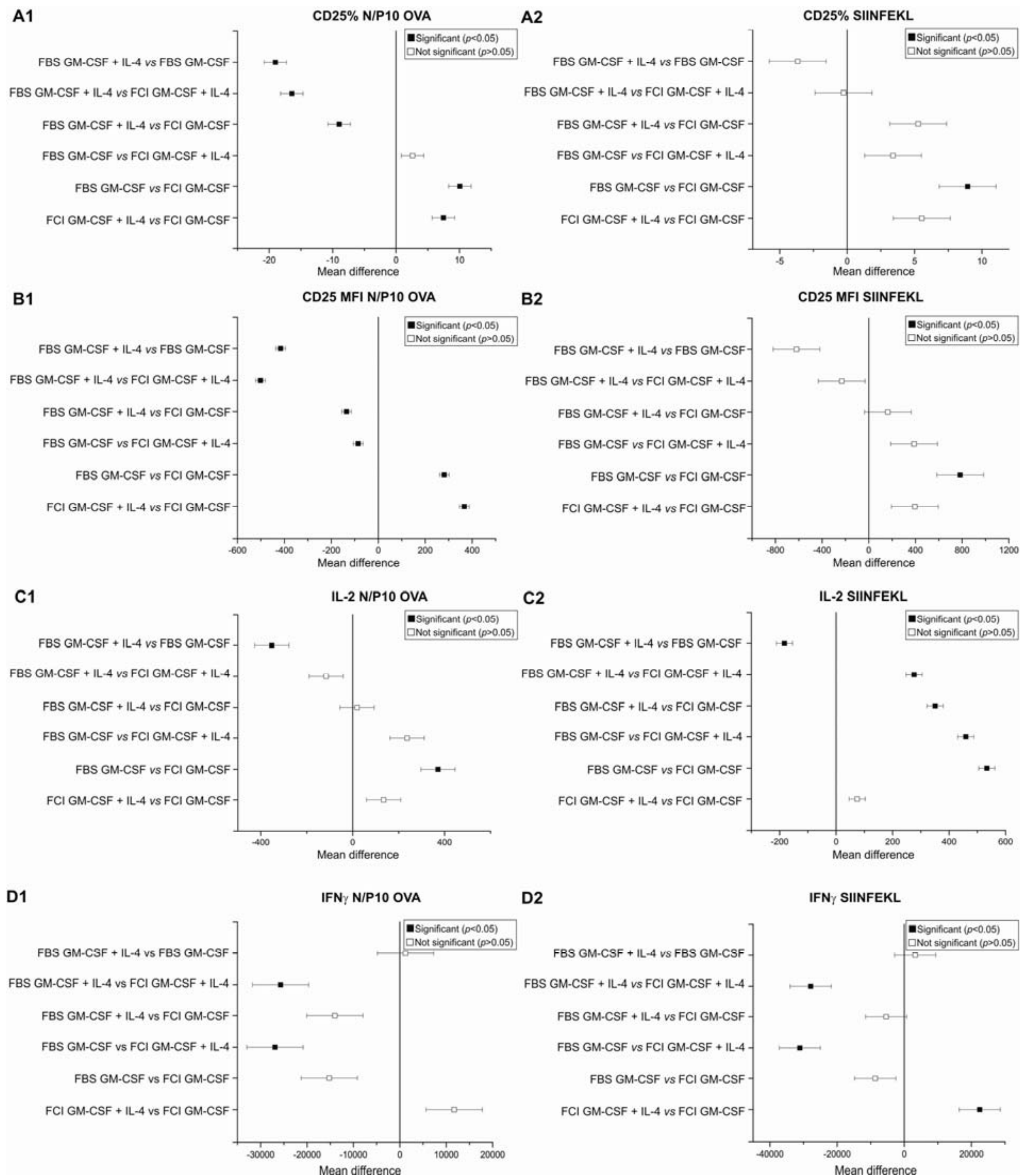

Figure S1. Statistical analysis of antigen-specific T cell activation by mRNA lipoplex transfected DCs. The results of the T cell activation experiments shown in Figure 5 were statistically analyzed with a one-way ANOVA with Bonferroni correction. Graphs show the mean difference with standard errors between the results obtained within different BM-DC cultures for: (A) the percentage of CD25 positive OT-I cells after stimulation with OVA-lipoplex transfected BM-DCs (A1) or SIINFEKL-loaded DCs (A2); (B) for the CD25 mean fluorescence

intensity on OT-I cells after stimulation with OVA-lipoplex transfected BM-DCs (B1) or SIINFEKL-loaded DCs (B2); for the secretion of IL-2 after stimulation with OVA-lipoplex transfected BM-DCs (C1) or SIINFEKL-loaded DCs (C2); (D) for the secretion of IFNγ after stimulation with OVA-lipoplex transfected BM-DCs (D1) or SIINFEKL-loaded DCs (D2)

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