pDNA-lipoplexes engrafted with flagellin-related peptide induce potent immunity and anti-tumour effects

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ABSTRACT

Complexes of cationic lipids and DNA (lipoplexes) are widely used for non-viral gene delivery and DNA vaccine development, but cationic lipids are toxic and promote non-specific interactions with cells, leading to poor efficacy. Near-neutral lipoplexes, on the other hand, can obviate toxicity, but a convenient means to target them to specific cells such as dendritic cells (DCs) has been lacking. Here, we show that a His-tagged flagellin-derived peptide (denoted 9Flg), previously reported to promote binding of liposomal antigen to TLR5-expressing cells, can be used to target near-neutral pDNA-lipoplexes incorporating the chelator lipid NTA_3-DTDA (3(nitrilotriacetic acid)-ditetradecylamine) to DCs and other antigen-presenting cells (APCs). Thus, we show that pDNA-lipoplexes engrafted with 9Flg target pDNA to APCs *in vitro* and *in vivo*. Following i.v. administration, radiolabelled 9Flg-lipoplexes exhibited increased accumulation in spleen, lung and liver. Vaccination of C57BL/6 mice with 9Flg-lipoplexes containing either pcDNA3.1-SIIN (pSIIN) or a Kunjin virus replicon-based vector (pKUN), each encoding the epitope $OVA_{257-264}$ (SIINFEKL), induced Ag-specific T cell priming, and elicited strong cellular immunity as reflected by a marked increase in the number of Ag-responsive IFN-γ-producing $CD8^+$ T cells. Importantly, compared to i.m. injection of these SIINFEKL-encoding pDNAs in naked form, the i.v. administration of pSIIN or pKUN in 9Flg-lipoplexes to C57BL/6 mice induced a significantly more potent anti-tumour response in the B16-OVA melanoma tumour model. The targeting of near-neutral 9Flg-lipoplexes bearing pDNA encoding tumour antigens to TLR5 on APCs, therefore, is a powerful approach for developing more effective DNA vaccines and immunotherapies.

1. Introduction

The induction of a strong cytotoxic T cell (CTL) response is crucial for eliciting effective cell-mediated immunity and the immune regression/prevention of tumours [1]. A number of studies show that the injection of naked antigen-(Ag)-encoding plasmid DNA (pDNA) into skeletal muscle can elicit Ag-specific CTL responses in mice [2,3]. The efficacy of such naked pDNA as a vaccine, how-

ever, is often very poor when injected intravenously, due to rapid degradation of the DNA by serum nucleases, poor DNA targeting or cell-specific delivery [such as to antigen presenting cells (APCs)], and poor cellular uptake and expression of the DNA [4,5]. A variety of methods have been developed, therefore, to load pDNA into suitable nano-carriers that can shield the pDNA from the harsh *in vivo* conditions and yet allow its efficient delivery to the cells of interest. Due to their superior transfection efficiency, the majority of nucleic acid-based vaccines in clinical trials have involved viral vectors. However, such vectors pose safety concerns due to possible mutagenic integration of the viral DNA into the host genome [6] and the induction of unwanted immune responses against the viral vector [7]. An approach that can avoid or minimize these risks is the use of pDNA complexed with lipids or agents designed to target the DNA to specific target cells *in vivo* [8].

Complexes of liposomes and DNA (hereafter referred to as lipoplexes) used for transfection either *in vitro* or *in vivo* typically contain a large proportion of different cationic lipids

(e.g. DOTAP and DOTIM) to promote condensation of the DNA [9,10], and helper lipids (e.g. cholesterol and DOPE), to promote lipoplex stability and intracellular DNA release [11]. Positively charged agents such as protamine [12] and cationic polymers (e.g. polyethyleneimine) [13] also have been used as DNA condensing agents. Cationic/positively charged lipoplexes formed in this way, however, are non-targeted and interact strongly with serum and other blood components, leading to the induction of unwanted non-specific effects, e.g., apoptosis [14] and cytotoxicity [15,16]. Importantly, some of these difficulties can be addressed by using lipoplexes containing sterically stabilizing lipids (e.g. phosphatidyl-ethanolamine-polyethylene glycol-2000 (PE-PEG$_{2000}$) and anionic pH-sensitive lipoplexes exhibiting prolonged circulation time *in vivo* and permit site-specific delivery of the pDNA [17]. Such formulations can trigger DNA release after internalization [18,19], and lead to increased transgene expression [20]. A major challenge of this approach, however, has been the difficulty in achieving site-specific delivery.

The ability to preferentially deliver pDNA to APCs *in vivo* is generally considered a major goal for effective DNA vaccination; but so far this has been met with only limited success. We recently showed that DODAP-containing near-neutral lipoplexes that incorporate the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA$_3$-DTDA) to permit anchoring of His-tagged targeting peptides, can be used effectively for the transfection of cells *in vitro* [8]. We also found that liposomal Ag engrafted with 9Flg peptide can be used to target Ag to TLR5 on APCs, thereby enhancing Agspecific and anti-tumour immunity [21]. In the present work we explored the potential of using 9Flg-lipoplexes containing pDNA encoding the MHC class I immunodominant epitope (SIINFEKL, C57BL/6 mice H-2^b) in the model Ag OVA, as an indicator of targeted *in vivo* transfection. Along with a standard pcDNA3.1-based vector encoding SIINFEKL (pSIIN), we used a SIINFEKL-encoding Kunjin virus replicon-based vector (pKUN), which can potentially enhance the response by amplification of viral RNA inside the cell [22]. Our results show that pDNA-lipoplexes engrafted with 9Flg bind to APCs, and that i.v. administration of the lipoplexes elicits Agspecific immunity and potently inhibits tumour growth/metastasis in the B16-OVA murine melanoma tumour model.

2. Materials and methods

2.1. Reagents

The phospholipid 1,2-dioleoyl-phosphoethanolamine (DOPE), as well as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Also, 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000] (PE-PEG$_{2000}$) were obtained from Avanti Polar Lipids (Alabaster, USA). The fluorescent lipid Oregon-Green-488-(1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) (OG$_{488}$-DHPE) was purchased from Invitrogen (Eugene, Oregon); and [4-^{14}C]-cholesterol was purchased from Perkin Elmer (Glen Waverley, Vic., Australia). The chelator-lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA$_3$-DTDA) was produced in the Research School of Chemistry (ANU) as described previously [23]. Paraformaldehyde was from BDH Chemicals (Kilsyth, Victoria, Australia). RPMI-1640 (Invitrogen) medium was obtained from the Media Unit, John Curtin School of Medical Research (ANU).

2.2. Plasmid DNA

The vector pcDNA3.1D/V5-His-TOPO with an insert encoding NP-SIMNFEKL-eGFP (pSIIN) was kindly provided by Dr. David

Tscharke (Research School of Biology, ANU). pKUNMpt, a Kunjin virus based vector (pKUN) encoding for different CTL epitopes one of which is SIINFEKL [24] was provided by Prof. Andreas Suhrbier and Prof. Alexander Khromykh (School of Chemistry and Molecular Biosciences, University of Queensland, Australia). The vector pEGFP-N1 was obtained from Becton Dickinson (North Ryde, NSW, Australia). Competent DH5α *Escherichia coli* were transformed separately with plasmids, and the plasmids were then separately amplified in the bacteria and purified using the PureYield Plasmid MaxiPrep System from Promega (Sydney, Australia). Purified plasmid DNA was eluted in nuclease-free water and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Peptides

The flagellin-related peptide used in this study (9Flg, of sequence HHHHHHHHHHHH-GSGSG-INNNLQRVRELAVQSANSTNSQSDLDS) was designed and produced as described previously [21]. This peptide contained a sequence corresponding to amino acids 85–111 of the 494-amino acid mature Flagellin FliC from *Salmonella typhimurium* (GeneBank Accession No. D13689); and also contained a short spacer (–GSGSG–) to reduce steric hindrance, and a stretch of 12 histidines to enable engraftment of the peptide onto lipoplexes. A 12 × His peptide containing a long spacer sequence (12 × His-GSGSGSGSGSGSGSGSGSGSGS) but no targeting sequence and referred to as p49 was used as a non-targeted (control) peptide to reduce non-specific binding of Ni-NTA$_3$-DTDA-liposomes to cells. All peptides were synthesized and HPLC purified by the Biomolecular Resource Facility, JCSMR (ANU). Stock solutions of peptides were prepared in distilled water, stored at −20 °C, and then thawed and vortexed immediately before use.

2.4. Cells

The immortalized murine dendritic cell line DC2.4 and the human embryonic kidney cell line HEK-293 stably transfected to express mouse H2^b were kindly provided by Dr. David Tscharke (Research School of Biology, ANU); the cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 0.1 mg/ml penicillin–streptomycin–neomycin (referred to as complete medium). The highly metastatic murine melanoma B16-OVA [C57BL/6 (H-2b)], an OVA-secreting tumour cell line, was a kind gift from Dr. Mark Hulett (Division of Molecular Bioscience, JCSMR; these cells were cultured at 37 °C in an atmosphere of 5% CO$_2$ in complete RPMI 1640 medium with added selection antibiotic Geneticin (Invitrogen; 0.5 mg/ml).

2.5. Mice

Female C57BL/6 (H-2^b) mice at 4–7 weeks of age were obtained from the Animal Resource Facility (Perth, WA). The mice were kept in the ANU Animal Facility and used at 6–8 weeks of age in accordance with protocols approved by the Australian National University Animal Experimentation Ethics Committee.

2.6. Preparation of liposomes

Stock solutions of NTA$_3$-DTDA were prepared in PBS and stored at −20 °C. Ni^{2+} was added to NTA$_3$-DTDA immediately prior to use. Stock solutions of all other lipids were prepared in ethanol, and stored at −80 °C. Lipids were mixed in the ratio DODAP:DOTAP:DOPE:cholesterol:PE-PEG$_{2000}$:Ni-NTA$_3$-DTDA (44:2:17:30:5:2 mol%) and dried under a stream of nitrogen

gas. Liposomes were produced by suspending the lipids in ddH_2O by sonication. To produce radiolabelled liposomes/lipoplexes, trace $[4-^{14}C]$-cholesterol (equivalent to 0.8 μCi for each dose of injection) was added to the lipid mixture and incorporated into liposomes by sonication in a bath sonicator for 5 min at maximum amplitude.

2.7. Preparation of pDNA-lipoplexes

Lipoplexes were prepared using a protocol established previously [8]. Plasmid DNA was added (10:1 lipid:pDNA, w/w) to an acidified suspension of liposomes (4 mM lipid in 8.3 mM citrate buffer pH 4.5). The liposome/DNA were mixed by pipetting and lipoplexes were allowed to form over 30 min at room temperature. The pH of the lipoplex suspension was neutralized by the addition of Na_2HPO_4 (final concentration 8 mM) and a 1/10 dilution of 10× PBS, before engrafting the lipoplexes with the indicated His-tagged peptide, by addition of the peptide and incubating for 30 min at room temperature.

2.8. Assaying binding of lipoplexes to cells and flow cytometry

DC2.4 cells (1×10^5 cells/condition) suspended in RPMI supplemented with 50% FCS and 20 mM HEPES (pH 7.5) were incubated with lipoplexes pre-engrafted with 9Flg or p49. The cells (~300 μl volume) were incubated with lipoplexes in 500 μl Eppendorf tubes for 1 h at 37 °C with continuous slow rotation [8]. After the incubation, the cells were washed thrice in PBS, fixed with 2% paraformaldehyde in PBS, and analyzed for cell-bound liposome fluorescence by using a flow cytometer (Becton Dickinson LSRII). Typically, a total of 10,000 or 30,000 cells were counted; dead cells were excluded by appropriate gating based on the forward versus side scatter of the cells. For detection of intracellular IFN-γ (see below), a minimum of 200,000 cells was counted for each condition.

2.9. Assaying transgene expression by intraperitoneal cells in vivo

Mice were injected i.p. (at time 0 and then again after 16 h) with either PBS or with pSIIN-lipoplexes engrafted with either p49 or 9Flg. For these experiments each dose of engrafted lipoplexes was administered in a volume of 800 μl containing 100 μg pDNA, 1 mg lipid and 50 nmol peptide. Mice were sacrificed 24 h after the first injection and peritoneal cells depleted of RBC were prepared as described previously [25]. The cells were then stained with APC-conjugated H-2K^b-SIINFEKL mAb, washed thrice in PBS, fixed with 2% paraformaldehyde in PBS, and analyzed for EGFP fluorescence or mAb staining by flow cytometry; a total of 30,000 or 50,000 cells were counted.

2.10. Biodistribution of peptide-targeted lipoplexes

Peptide-engrafted lipoplexes were prepared from liposomes containing a trace amount of $[4-^{14}C]$-cholesterol (equivalent to 0.8 μCi for each dose of injection) and injected i.v. into C57BL/6 mice (20 μg pDNA). At 24 h post-injection, mice were killed by CO_2 asphyxiation. Tissues were harvested, weighed and solubilized in 1 ml of a solution containing 0.5 M sodium hydroxide, 150 mM sodium chloride and 2% SDS. Solubilization was assisted by heating at 60 °C for 24 h. The solubilized tissue (0.3 ml) was added to 3 ml Emulsifier-Safe scintillant (Perkin-Elmer), and radioactivity (dpm) measured using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, USA). The results were expressed as the percentage of the injected dose per gram of tissue (%ID/g).

2.11. Measurement of cell proliferation

Splenocytes were prepared from vaccinated mice exactly as described [25]. For measurement of proliferation the splenocytes

were incubated with soluble OVA (100 μg/ml) in a 96-well flat-bottom plate in complete RPMI medium supplemented with IL-2 (50 IU/ml). After 72 h cell ^3H-thymidine was added and proliferation was measured using a standard ^3H-thymidine incorporation assay as described [25]. For each condition three mice were vaccinated and the extent of cell proliferation for each mouse was measured in triplicate.

2.12. Intracellular staining for IFN-γ

A total of 1×10^6 splenocytes from each mouse was stimulated for 2 h with 1 μg/ml SIINFEKL; and a 1 in 1000 dilution of BD GolgiStop™ (BD Biosciences) was then added and the cells incubated for another 3 h, before staining with anti-CD8-PE (clone 53–6.7; Pharmingen), and intracellularly with anti-IFN-γ-Alexa Fluor®-647 (clone XMG1.2; Pharmingen) as described [25]. Flow cytometric data were acquired in BD LSR II and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). CD8+ cells were gated from live cells to give the IFN-γ+ CD8+ cells in a two-colour dot plot; for each condition the percentage of cells was obtained by subtracting the background (obtained from conditions for which PBS was added instead of SIINFEKL peptide). Data from three mice was collected to determine the extent of IFN-γ production for each different vaccination condition.

2.13. Vaccination of mice and tumour therapy

Typically, female mice (4–6 weeks of age) were vaccinated: with 200 μl of the vaccine preparation by i.v. tail vein injection, or with 100 μl of the vaccine preparation by i.m. injection into the right upper leg flank. For induction of functional immune responses mice were injected i.v. on days 0, 5 and 14. Each dose of the lipoplexes contained 30 μg pDNA, 300 μg lipid and 15 nmol peptide. All conditions including controls contained the same amount of pDNA and each dose was administered either i.v. or i.m. as indicated. To assess the induction of immune responses, mice were sacrificed on day 21.

For tumour studies, mice were first inoculated with B16-OVA cells (2×10^5 cells) in RPMI medium (without serum) by i.v. (tail vein) injection on day 0. Different groups of tumour-inoculated mice were then vaccinated either: i.v. with p49- or 9Flg-lipoplexes on day 2 and day 7; or i.m. with naked pDNA on day 2, day 7 and day 14. For the vaccination with lipoplexes, the first administration (day 2) contained approx. 20 μg pDNA, 200 μg lipid and 10 nmol peptide; whereas the second administration (day 7) contained a quarter of these amounts (giving a total of 25 μg pDNA for these vaccinations). For the vaccination with naked pDNA three lots of 30 μg pDNA were administered i.m. (giving a total of 90 μg pDNA). To quantify tumour metastases the mice were sacrificed at day 21 after B16-OVA tumour challenge, and the number of tumour foci in the lungs were counted visually using a dissection microscope. For each condition a total of 5 mice was used to determine the efficacy of pDNA vaccination.

2.14. Statistical analyses

All data are represented as means ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's post-test was used for comparison of significance between 3 or more groups. Statistical analyses with p-value < 0.05 were considered to be statistically significant.

3. Results

3.1. Lipoplexes engrafted with 9Flg peptide bind to DCs

We recently established that the engraftment of liposomes with the flagellin-related peptide 9Flg can promote binding of

the liposomes to DCs and to other TLR5-expressing APCs both *in vitro* and *in vivo* [21]. We hypothesized, therefore, that NTA_3-DTDA-containing lipoplexes engrafted with 9Flg would also exhibit binding specificity towards DCs, facilitating expression of the incorporated transgene. To explore this, binding experiments were carried out in which tracer OG_{488}-DHPE containing NTA_3-DTDA-lipoplexes engrafted with either p49 (as control) or 9Flg peptide were incubated with DC2.4 cells for 1 h at 37 °C, before washing the cells and analysing them for OG-fluorescence by flow cytometry. The results indicate that whereas DC2.4 cells incubated with p49-lipoplexes (control) exhibited ∼6-fold increase in mean fluorescence intensity (MFI) above background, the increase in MFI of cells incubated with 9Flg-lipoplexes was ∼5.5-fold higher than for the control cells (Fig. 1A). This demonstrates that the engraftment of 9Flg peptide promotes binding of the lipoplexes to DCs.

### 3.2. Transfection of cells with pSIIN and pKUN induce MHC class I Ag presentation

To establish that the two pDNA constructs used in this work, could lead to the expression of the encoded transgene Ag, and processing and presentation of antigenic peptide on MHC class I molecules, HEK-293 cells stably expressing the mouse class I allele H-2K^b were transfected using Lipofectamine^{TM} 2000 (Invitrogen) with pSIIN, pKUN or with the pEGFP-N1 vector as control. After 48 h the cells were harvested and stained with mAb specific for OVA$_{257-264}$ (SIINFEKL) peptide bound to H-2K^b (H-2K^b-SIINFEKL, OVA Kb). Analysis of the cells by FACS indicated that the SIINFEKL epitope was expressed in the context of mouse MHC class-I. The results show that a substantial proportion (∼60.7%) of HEK-293-H-2K^b cells transfected with pEGFP-N1 expressed EGFP, but did not exhibit significant binding of H-2K^b-SIINFEKL mAb (Fig. 1B). Importantly, however, a significant proportion of HEK-293-H-2K^b cells transfected with pSIIN and pKUN (total of ∼18.44% and ∼9.28%, respectively) bound the mAb, indicating effective expression of the OVA$_{257-264}$ epitope in the context of MHC class I (Fig. 1B).

3.3. 9Flg-lipoplexes containing pSIIN promote transgene expression by peritoneal cells

To assess the ability of 9Flg-lipoplexes to promote DNA delivery and transgene expression in APCs *in vivo*, naïve C57BL/6 mice were injected i.p. at time 0 and again 16 h later with either PBS or Lipo-SIIN engrafted with either p49 or 9Flg. Peritoneal cells were collected 24 h after the first injection and assessed for transgene expression, by staining with APC-conjugated H-2K^b-SIINFEKL mAb and examining the cells for EGFP fluorescence and mAb staining by flow cytometry. As shown by the contour plots in Fig. 1C, the results indicate no significant transgene expression in peritoneal cells of mice injected with PBS or p49- Lipo-pSIIN as ∼0.03% cells were positive for both EGFP and SIIN-MHC-I expression. In contrast, ∼1.6% of peritoneal cells derived from mice injected with 9Flg-Lipo-pSIIN were positive for EGFP expression, and ∼0.58% of peritoneal cells were positive for both EGFP and SIIN-MHC-I complex expression indicating effective transgene expression by a proportion of peritoneal cells under these conditions.
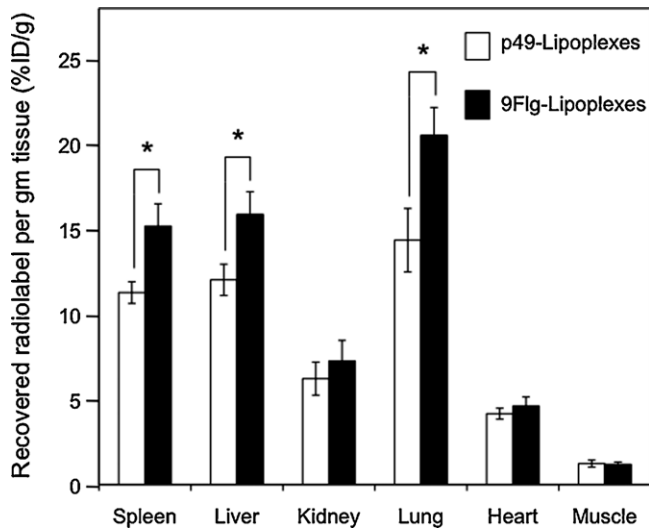
3.4. Biodistribution of 9Flg-lipoplexes

To determine the nature of the tissues being targeted after i.v. administration of p49- and 9Flg-lipoplexes into mice, biodistribution studies were carried out to determine the tissues in which the lipoplexes accumulated. Engrafted lipoplexes containing ^{14}C-cholesterol as tracer were prepared and injected i.v. into C57BL/6
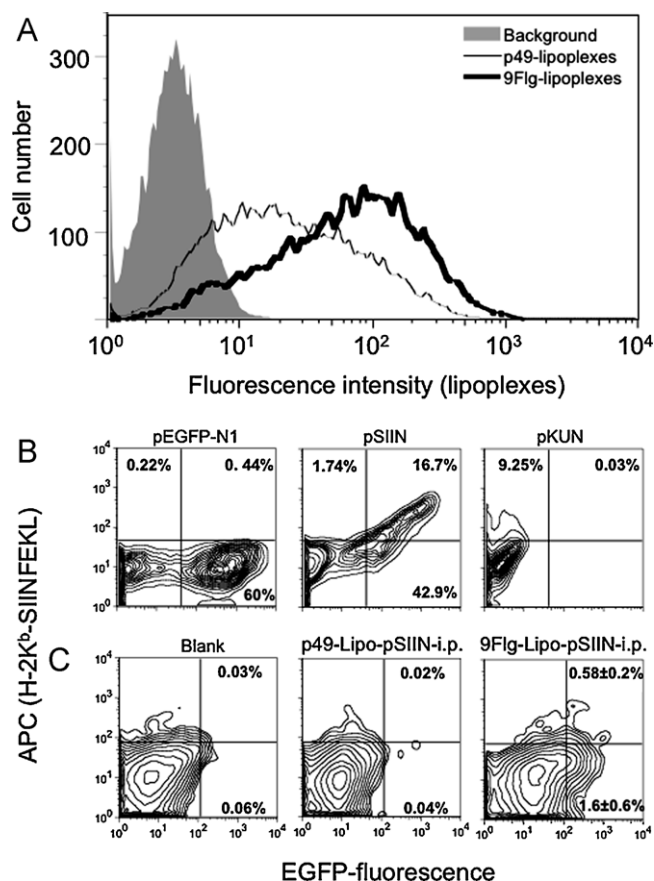
Fig. 1. 9Flg-engrafted lipoplexes bind to APCs and the encoded Ag is expressed and presented on APCs *in vivo*. Lipoplexes containing pSIIN and OG_{488}-DHPE as tracer were engrafted with either p49 or 9Flg peptide and then incubated with DC2.4 cells for 1 h at 37 °C. The cells were then washed and assessed for lipoplex binding by analysis of their OG_{488}-fluorescence using flow cytometry. The fluorescence profiles shown in (A) represent cells incubated with lipoplexes engrafted with p49-Control (thin line) and 9Flg peptide (thick line). The shaded histogram represents background fluorescence of DC2.4 cells not incubated with lipoplexes. Each fluorescence profile is a representative of three separate incubations of lipoplexes with cells. For (B), HEK-293 cells stably expressing the mouse class I allele H-2K^b, were transfected with pEGFP-N1 (control), pSIIN or pKUN (as indicated) using Lipofectamine^{TM} 2000. After 48 h, the cells were harvested and stained with APC-conjugated H-2K^b-SIINFEKL mAb. The contour plots shown in (B) represents the EGFP expression by the cells for pEGFP-N1 and pSIIN (FITC, *X*-axis) and mAb-staining (APC, *Y*-axis). Each fluorescence profile is a representative from three independent experiments. For (C) naïve C57/BL6 mice were injected i.p. initially at time 0 and again 16 h later with PBS (sham), and with the pSIIN-lipoplexes engrafted with either p49 (control) or 9Flg peptide. Peritoneal cells were then collected from the mice 24 h after the first injection, and the cells stained with APC-conjugated H-2K^b-SIINFEKL mAb. The contour plots shown in (C) represents EGFP expression by the cells (FITC, *X*-axis) versus mAb staining (APC, *Y*-axis). Each contour plot is representative of 4 independent experiments and the mean percentage (%) of gated live cells that are double positive for both EGFP expression and mAb staining is shown.

mice, and distribution of the radiolabel in the different tissues was determined 24 h after injection. The results in Fig. 2 show that the proportion of radiolabel recovered in the spleen (15.2 ± 0.6%ID/g), liver (15.9 ± 0.9%ID/g) and lungs (20.5 ± 1.0%ID/g) was significantly greater (20–30% higher) in mice injected with 9Flg-lipoplexes, compared to mice injected with p49-lipoplexes. The distribution of radiolabel in the kidney and heart was not significantly different between the mice injected with 9Flg-lipoplexes and the mice injected with p49-lipoplexes (as control). In addition, muscle tissue showed a low level of radiolabel accumulation (∼1%ID/g) for both the p49-control and 9Flg-targeted formulations, indicating a significantly lower level of lipoplex accumulation (Fig. 2).

Fig. 2. Tissue biodistribution of peptide-engrafted lipoplexes after intravenous administration. Lipoplexes incorporating radiolabel [4-^{14}C]-cholesterol as tracer were prepared and engrafted with either p49-control or 9Flg peptide before injecting i.v. into different groups of 5 C57BL/6 mice. After 24 h, tissues were harvested from the mice, and the amount of radioactivity associated with each tissue was measured, expressed as a proportion of the injected radiolabel/gram tissue, and the results presented as a bar graph. Results represent the mean ± SEM from six mice per condition; *denotes $p < 0.05$, relative to the respective controls as indicated, using ANOVA with Bonferroni post hoc test.

3.5. Vaccination with pSIIN- and pKUN-lipoplexes engrafted with 9Flg peptide elicit Ag-specific immunity

Lipoplexes containing pSIIN or pKUN and engrafted with 9Flg peptide were also examined for their ability to induce Ag-specific immunity in C57BL/6 mice, utilizing SIINFEKL as the DNA-encoded Ag. In recent studies we showed that the vaccination of mice with Ag-containing liposomes engrafted with 9Flg could elicit potent Ag-specific responses [21]. In analogous studies, we examined the ability of 9Flg-lipoplexes to induce immunity to the encoded SIINFEKL-Ag, thus enabling us to assess the potential of this approach for development of DNA vaccines. Since the majority of DNA vaccines utilize naked DNA, which is typically administered intramuscularly (skeletal muscle), we used i.m. administration of naked pDNA as the positive control, and compared this with the same amount of pDNA administered via the i.v. route in the form of 9Flg-lipoplexes. This approach enabled us to assess the effect of a different route of administration as well as the effect of including the 9Flg targeting peptide, on the induction of Ag-specific immunity to the DNA-encoded Ag. Experiments were designed to contain two identical sets of conditions each employing either the pSIIN or pKUN pDNA constructs.

3.5.1. T cell priming

Different groups of 3, C57BL/6 mice were vaccinated on days 0, 5 and 14 with PBS (i.v.), naked pDNA (i.v. and i.m.), or with lipoplexes containing either pSIIN or pKUN; and engrafted with either p49 or 9Flg (i.v.). pSIIN and pKUN were used either in naked form (naked pDNA), or were first incorporated into lipoplexes, before engrafting with either control (p49) or 9Flg peptide and administering to mice. At day 21 splenocytes were prepared from the spleens of vaccinated mice, and the splenocytes stimulated *in vitro* with OVA (100 μg/ml) before assessing cell proliferation at 72 h by standard ^{3}H-thymidine incorporation assay; alternatively, the splenocytes were stimulated with SIINFEKL peptide to assess Ag-specific production of IFN-γ.

As shown in Fig. 3, splenocytes from negative control mice, vaccinated i.v. with PBS, pSIIN (i.v.), pKUN (i.v.), p49-lipoplex-pSIIN or
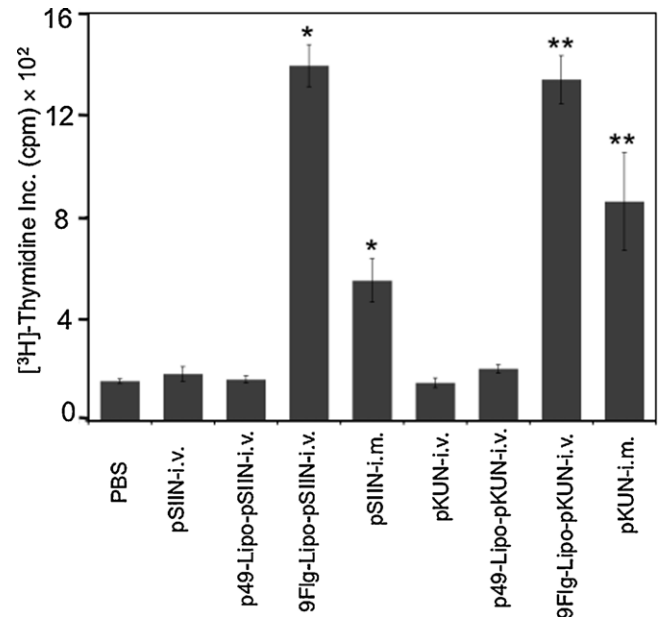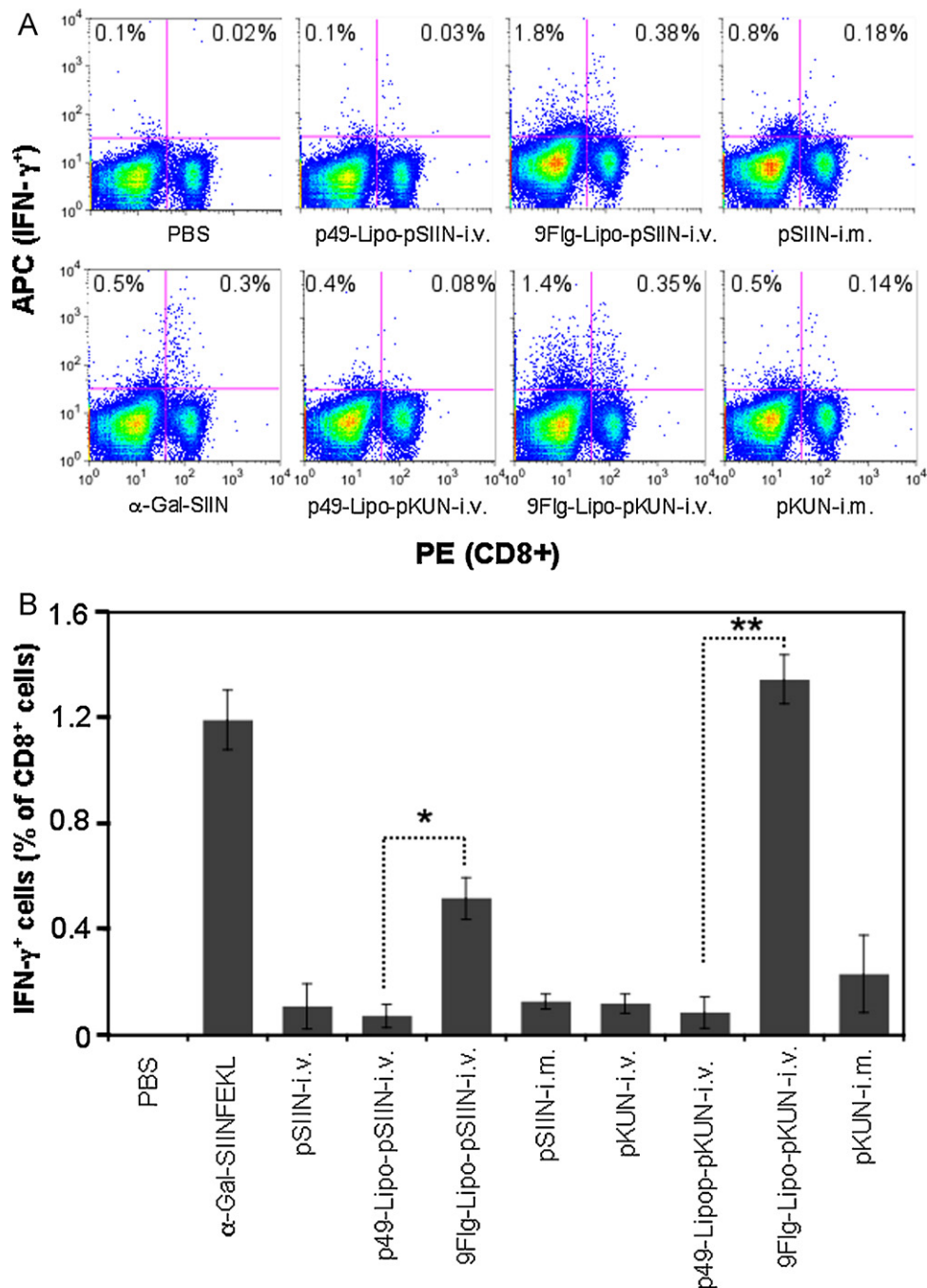

Fig. 3. Vaccination with 9Flg-lipoplexes induces Ag-specific T cell priming. Groups of three naïve C57BL6 mice were vaccinated i.v. on days 0, 5 and 14 with PBS (i.v.), with either naked pDNA (i.v. or i.m.), or with lipoplexes engrafted with either p49 or 9Flg (i.v.) as indicated. The two different pDNA constructs used were: pSIIN and pKUN. On day 21, splenocytes were prepared from each vaccinated mouse and assessed for the induction of OVA-specific T cell priming. A total of 5×10^5 cells were incubated (3 h, 37 °C) in the presence of OVA (100 μg/ml) in complete RPMI-1640 medium plus 50 IU/ml IL-2. Cell proliferation was assessed after 72 h by measurement of the uptake of ^{3}H-thymidine. The proliferative response of splenocytes from each mouse was measured in triplicates. The, bar graph represents mean cpm ±SEM of nine cultures for each condition; *denotes $p < 0.001$ relative to p49-Lipo-pSIIN-i.v.; and **denotes $p < 0.01$ relative to p49-Lipo-pKUN-i.v., as indicated, using ANOVA with Bonferroni post hoc test.

p49-lipoplex-pKUN exhibited little if any proliferative response. In contrast to the i.v. injection of pSIIN and pKUN, the i.m. injection of these two constructs (treated here as positive controls), induced ~3.2- and 5-fold higher proliferative response (Fig. 3). However, splenocytes from mice vaccinated with 9Flg-lipoplexes-pSIIN or 9Flg-lipoplexes-pKUN both showed levels of proliferation which were ~8-fold higher, than the average proliferative response of the negative controls; and ~2.5- and ~1.5-fold higher, respectively, than that of the pSIIN and pKUN injected i.m. as naked DNA.

3.5.2. Ag-specific CD8$^+$ T cell response

Splenocytes from the mice vaccinated above also were used to assess OVA-specific CD8$^+$ T cell immunity. Splenocytes were stimulated for 5-h with 1 μM SIINFEKL (class I immuno-dominant epitope in OVA for H-2K^b) peptide, also in the presence of monensin for the last 3 h. After the incubation, splenocytes were stained with CD8 mAb, fixed, permeabilized, and then stained intracellularly with IFN-γ mAb, before analyzing the cells for fluorescence by FACS. The two-colour dot plots in Fig. 4A are representative of three independent experiments showing the percentage of CD8$^+$ and CD8$^-$ splenocytes producing IFN-γ in response to SIINFEKL peptide; with the average of Ag-responsive IFN-γ$^+$ CD8$^+$ T cells from three experiments being shown in Fig. 4B. The results indicate that no significant IFN-γ response was induced in splenocytes from negative control vaccinated mice (where <0.2% of CD8$^+$ T cells produced IFN-γ). However, in contrast to these controls, splenocytes from mice vaccinated with 9Flg-lipoplexes-pSIIN and those from mice vaccinated with 9Flg-lipoplex-pKUN showed substantial increases (~0.5% and ~1.3%, respectively) in the proportion of CD8$^+$ T cells that were induced to produce IFN-γ, in response to SIINFEKL Ag-specific stimulation (Fig. 4B).
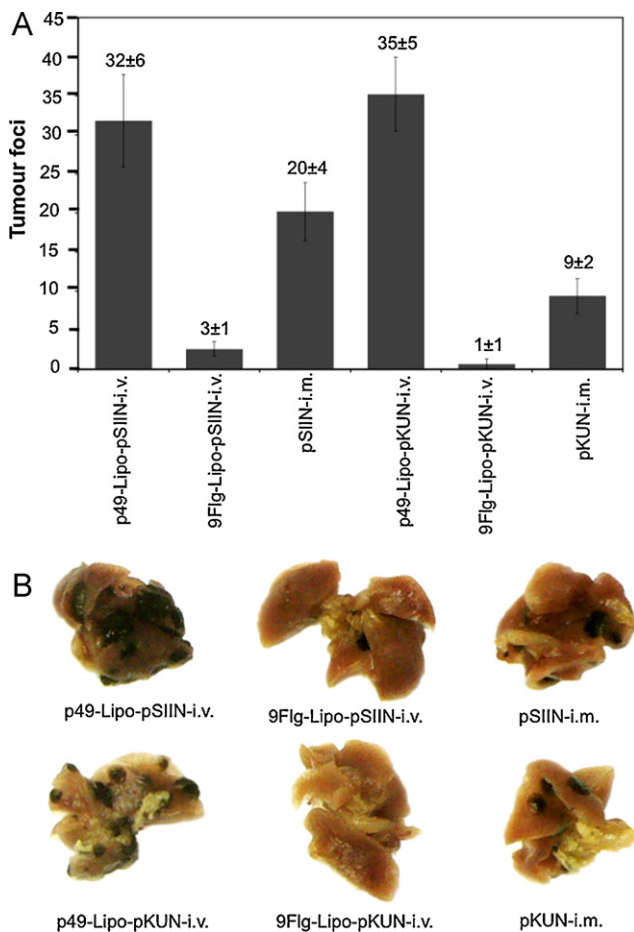
Fig. 4. Vaccination with 9Flg-lipoplexes primes Ag-specific CD8^{+} T cells. Splenocytes from mice vaccinated as detailed in the legend to Fig. 3 were also assessed for production of IFN-γ in response to stimulation with SIINFEKL peptide. The splenocytes (1×10^6) were incubated in the absence or presence of SIINFEKL (1 μg/ml) for 5 h, with monensin being added after the first 2 h incubation. The cells were then washed, stained with PE-labelled mAb to CD8, washed again, fixed, permeabilized and stained intracellularly with APC-labelled mAb to IFN-γ before analysis by flow cytometry. Splenocytes from mice vaccinated with a mixture of α-galactoceramide (α-galcer, 1 μg) and SIINFEKL (0.1 mg) 7 days prior to the *in vitro* assay were used as positive control. The two-colour dot plots in (A) show IFN-γ production by splenocytes from the vaccinated mice obtained from one representative experiment; the percentage of CD8^{+} and CD8^{−} splenocytes producing IFN-γ (relative to the total number of live-gated cells) is indicated in the respective quadrant. For (B), the bar graph shows the percentage of IFN-γ-producing cells in the CD8^{+} gated population for the conditions indicated, as determined by analysis of the respective two-colour dot plot and subtracting the background staining (of cells incubated with PBS) for each condition. The resultant difference corresponding to the IFN-γ response was calculated for three independent experiments and each result in (B) represents the mean ± SD from three experiments; * denotes $p < 0.05$ relative to p49-Lipo-SIIN-i.v.; and ** denotes $p < 0.01$ relative to p49-Lipo-KUN-i.v., using ANOVA with Bonferroni post hoc test.

3.6. Vaccination with 9Flg-lipoplexes induce potent anti-tumour immunity

The ability of 9Flg-lipoplexes to induce anti-tumour immunity was examined utilizing the murine tumour model, B16-OVA melanoma. Following i.v. inoculation, B16-OVA cells metastasize initially to the lungs and then to other major organs in syngeneic C57BL/6 mice. Experiments were carried out in which groups of 5 naïve C57BL/6 mice were first inoculated with B16-OVA cells (2×10^5 cells) by i.v. injection on day 0. The mice were then vaccinated: either by i.v. injection on days 2 and 7 with lipoplexes engrafted with p49 or 9Flg; or with PBS (i.v.) or naked pSIIN and

Fig. 5. Vaccination with 9Flg-lipoplexes elicits robust anti-tumour immunity. Naive C57/BL6 mice were inoculated with 2×10^5 B16-OVA cells by i.v. injection (day 0). Different groups of five inoculated mice were then vaccinated: with lipoplexes engrafted with p49 or 9Flg by i.v. injection on days 2 and 7; or with either PBS (i.v.) or naked pDNA (i.m.) on days 2, 7 and 14. The pDNA constructs used were pSIIN and pKUN, as indicated. At day 21, the mice were sacrificed, the lungs removed and the number of tumour foci counted with an inverted microscope to quantify tumour metastases. The bar graph in (A) shows the mean number of tumour foci in the lungs for each group of mice (as indicated); with representative images of the lungs from each group being shown in (B).

pKUN (i.m.) on days 2, 7 and 14. On day 21, the mice were sacrificed, lungs were removed and the number of tumour foci in the lungs counted to quantify the anti-tumour response.

As shown in Fig. 5, vaccination with p49-lipoplexes containing either pSIIN or pKUN offered little if any protection against challenge with the B16-OVA tumour. Similarly, the effects of i.v. injection of either pSIIN or pKUN in naked form were not significant (not shown). However, as shown in the bar graph (Fig. 5A), mice treated with i.m. injection of naked pSIIN and pKUN induced a moderate anti-tumour response with the lungs bearing an average of 20 ± 4 and 9 ± 2 tumour foci, respectively. In contrast, mice vaccinated with 9Flg-lipoplexes-pSIIN and 9Flg-lipoplexes-pKUN exhibited a robust anti-tumour response, with the lungs from these groups containing an average of 3 ± 1 and 1 ± 1 tumour foci, respectively. This demonstrates that the i.v. injection of 9Flg-lipoplexes is highly effective at inducing anti-tumour responses in this system. For comparison, differences in the appearance of the lungs from mice in the different treatment groups are shown in the representative images in Fig. 5B, where it can be seen that the lungs from mice vaccinated with 9Flg-lipoplexes are largely tumour free.

4. Discussion

The present work demonstrates that near-neutral lipoplexes containing pDNA and engrafted with a TLR5-specific targeting peptide can be used effectively to deliver pDNA to APCs both *in vitro* and *in vivo*. Moreover, such targeted lipoplexes can elicit Ag-specific immunity in mice with the results showing that the approach is ideal for the development of novel DNA vaccines.

Cationic liposomes and lipoplexes are widely used for gene delivery, but such agents have a tendency to aggregate in serum, leading to unwanted toxicity and inefficient delivery of the DNA to target cells [14–16]. To address these difficulties we recently developed a near-neutral formulation containing the ionisable lipid DODAP. This lipid is cationic at pH <6.6 (enabling condensation/incorporation of pDNA at acidic pH), but is uncharged at neutral or physiological pH (reducing non-specific binding). These attributes, coupled with inclusion of a sterically stabilizing lipid (e.g. PE-PEG$_{2000}$) in the formulation, markedly reduces the ability of lipoplexes to interact non-specifically with cells [8]. Also pertinent is that the inclusion of DOPE in the lipid mixture promotes the intracellular release of pDNA from lipoplexes (after internalization and acidification in endosomes) [26,27]; and that inclusion of the chelator lipid NTA$_3$-DTDA permits engraftment of His-tagged moieties for targeting to specific cells [8]. Using lipoplexes made from a similar lipid formulation we recently showed by performing Zetasizer measurements that the engraftment of peptide results in a slight increase in particle size between non-engrafted and engrafted lipoplexes (diameter 164 ± 1 and 255 ± 12 nm, respectively), but that this is the same for both targeting and non-targeting peptide. Moreover, there was no significant difference in Zeta potential between non-engrafted and engrafted lipoplexes (-18 ± 2 and -19 ± 3 mV, respectively) [8]. For peptides like p49 and 9Flg which do not bear a strong electric charge therefore, observed differences in the distribution and efficacy of the engrafted lipoplexes can be expected to result from differences in engrafted targeting moiety, and not from the engraftment or non-engraftment of peptide.

Our initial experiments indicated that compared to p49-lipoplexes, 9Flg-lipoplexes bound substantially more strongly to DC2.4 cells (Fig. 1A), consistent with an ability to target the lipoplexed pDNA to these cells. Furthermore, experiments in which lipoplexes were injected i.p. into mice showed that while no transgene expression could be detected in peritoneal cells of control mice injected either with PBS or p49-Lipo-pSIIN, a significant proportion of peritoneal cells from mice injected with 9Flg-Lipo-pSIIN were positive for both EGFP (reporter gene for pSIIN) and SIINFEKL-MHC-class I expression (Fig. 1C). These findings are consistent with 9Flg-lipoplexes being able to bind peritoneal APCs *in vivo*, and importantly, elicit the expression of the pDNA being targeted. The ability of the pDNA-encoded Ag to enter MHC class-I restricted pathway of Ag presentation also was explored in HEK-293-H-2K^b cells transfected with pSIIN or pKUN using Lipofectamine^{TM}-2000. The results show that a substantial proportion of HEK-293-H-2K^b cells transfected with either pSIIN or pKUN bound H-2K^b-SIINFEKL mAb, confirming introduction of the epitope into the MHC class I presentation pathway (Fig. 1B).

An important parameter in the development effective liposome-based DNA vaccines is the extent to which lipoplexes accumulate in major organs such as spleen, lung and liver [28,29]. Incorporated ^{14}C-cholesterol has been used as an effective tracer in many short-term (~24 h) liposome tracking experiments see e.g. [30,31]. Importantly, lipoplexes of lipid formulation similar to that used for the present work were shown to exhibit excellent stability under conditions mimicking those found *in vivo*; namely, incubation of the lipoplexes for 48 h at 37 °C in the presence of 50% serum [8]. The highly hydrophobic nature of ^{14}C-cholesterol enables it to incorporate/associate with the hydrophobic lipid tails in lipoplexes, thus

making it well suited for use in tracking experiments. Biodistribution experiments performed using ^{14}C-cholesterol in the present work indicated that after i.v. injection of 9Flg-lipoplexes, the majority of the injected 9Flg-lipoplex dose could be recovered from the spleen, lung and liver, with the proportion of 9Flg-lipoplexes detected in these tissues being significantly greater than those in mice injected with p49-lipoplexes (Fig. 2). Since secondary lymphoid organs such as the spleen are enriched in APCs, the ability to direct the lipoplexes to spleen can greatly influence the efficacy of DNA administered as a vaccine. Interestingly, our results show that ∼15% of the i.v. injected 9Flg-lipoplex dose was sequestered by the spleen, which is substantially greater than that reported in a previous study using a non-targeted delivery system where <5% of the administered dose was found to accumulate in spleen [32]. Thus, whilst cationic charge can promote the binding and uptake of lipoplexes by APCs in spleen and other tissues, the greater accumulation of 9Flg-lipoplexes in the spleen observed in the present work (using 9Flg-lipoplexes which are not cationic) is attributable to the engrafted 9Flg peptide, which promotes binding of the lipoplexes to TLR5 on APCs [21]. Notably, in addition to spleen, APCs in the liver and lung also are purported to express TLR5, and thus at least some of the observed 9Flg-lipoplex accumulation observed in these tissues may be attributable to TLR5 expression [21].

A major finding from the present work is that the i.v. administration of 9Flg-lipoplexes exhibits the ability to prime Ag-specific T cells, including IFN-γ producing CD8^{+} T cells. Compared to splenocytes from mice vaccinated with control p49-lipoplexes, splenocytes from C57BL/6 mice vaccinated with either 9Flg-Lipo-pSIIN or 9Flg-Lipo-pKUN responded more strongly to an *in vitro* exposure to OVA, resulting in increased Ag-specific cell proliferation (Fig. 3). The ability of 9Flg-lipoplexes to prime effector T cell function was also explored. Notably, IFN-γ is a cytokine crucial for mediating Th1 immune responses and increased cytotoxicity; and these attributes are crucial for inducing effective anti-tumour immunity [33,34]. Our results indicate that compared to controls, splenocytes of mice vaccinated with 9Flg-Lipo-pSIIN and 9Flg-Lipo-pKUN, comprised of a significantly higher proportion of CD8^{+} T cells (∼0.5% and ∼1.3%, respectively) that produced IFN-γ in response to SIINFEKL-Ag (Fig. 4), demonstrating that APC-targeted pDNA-lipoplex vaccination can elicit a strong CD8^{+} T cell response. Importantly, the response observed herein (where the pDNA is targeted to APCs with peptide 9Flg) is comparable to that observed after i.m. injection of a 4–5-fold larger amount of pDNA in the form of non-targeted cationic lipoplexes [10]. This indicates that APC-targeted 9Flg-pDNA-lipoplexes have a greater potency with respect to the total amount of pDNA required for effective vaccination. Interestingly, vaccination with 9Flg-lipoplexes also increased the proportion of IFN-γ^{+}CD8^{-} cells (Fig. 4A). The IFN-γ^{+}CD8^{-} cells were not characterized in this work, but it is likely that a proportion of these cells represents NK cells, since splenic NK cells purported to produce high levels of IFN-γ [10].

DNA vaccines have been conventionally administered as naked DNA or as cationic lipoplexes via either the i.m. or s.c. route. Indeed, i.m. injection of naked pDNA in our experiments induced moderate levels of Ag-specific immunity. However, our results also show that 9Flg-lipoplexes administered i.v. elicits immune responses that are substantially more potent than those induced by vaccination with pDNA in naked form (see Figs. 4 and 5). This is a major advance, since the i.v. route can potentially allow a more efficient delivery of lipoplexes to APCs in the blood circulation as well as to those in more immunologically relevant sites such as the spleen, liver and lung.

A robust test of efficacy for a vaccine or immunotherapeutic agent is to assess its ability to promote protection against disease. Thus, to test the efficacy of 9Flg-lipoplexes, we first inoculated C57BL/6 mice by i.v. injection of syngeneic B16-OVA

melanoma cells, and then vaccinated the mice with the different pDNA preparations. Our results show that compared to vaccination with control p49-lipoplexes, vaccination with 9Flg-lipoplexes elicits potent anti-tumour responses, reflected by a dramatic reduction in the number of lung metastases (Fig. 5). Interestingly, a prime-boost strategy employing 9Flg-lipoplexes as the priming vaccine and OVA-containing 9Flg-liposomes to boost the 'priming' effect also resulted in the induction of a potent anti-tumour response, with the majority of the treated mice remaining 'tumour-free' after the treatment regime (not shown). The two pDNA constructs used in the present work, encode an epitope of OVA and are applicable for use in the B16-OVA tumour model. For clinical applications, pDNA encoding one or more tumour Ags relevant to the particular tumour/cancer could be used.

It is noteworthy that the amount of pDNA typically administered to induce anti-tumour immune responses in mice by vaccination with naked DNA is typically 50–100 μg per dose [35]. By contrast, in the present work strong anti-tumour responses were observed after administering a total of 25 μg of lipoplexed pDNA, reflecting a greater potency when using targeted 9Flg-lipoplexes. Vaccination of mice with equal amounts of pSIIN or pKUN DNA (in either *naked* or lipoplexed form) showed that pKUN elicits stronger responses compared to pSIIN (Figs. 4B and 5). This finding was not unexpected since replicon-based vectors can induce high levels of gene expression by virtue of the ability of the replicon RNA (transcribed from replicon cDNA) to amplify itself within cells [22]. Since i.m. vaccinations of naked pKUN DNA with doses as low as 0.1 μg have been reported to induce Ag-specific CD 8^{+} T cell responses in BALB/c (2^{d}) mice [24], it is conceivable that the amount of pKUN DNA used in the present work could have been substantially reduced without compromise to the response induced.

A major focus for pDNA-based DNA vaccination has been cell/tissue-specific targeting. To achieve this, numerous strategies for pDNA-targeting including surface modification of lipoplexes have been reported. For example, mannose-conjugated polyethyleneimine (mannose-PEI) has been shown to promote gene transfer in primary human DCs *in vitro*; but such lipoplexes apparently were not potent enough to activate T cells, since the vehicle also required incorporation of adenovirus particles to induce a T cell response [36]. Attempts to target DNA to mediate transgene expression in DCs also has seen the conjugation of receptor-specific targeting peptides to DNA; with one study showing efficient transfection *in vitro* after using a TNF-α-specific peptide to deliver conjugated DNA to DCs [37]. For *in vivo* use such vector-free DNA targeting approaches may exhibit reduced efficacy, however, due to possible degradation of the pDNA in serum – underscoring the advantage of employing targeted pDNA-lipoplexes.

An important feature of our APC-targeted 9Flg-lipoplexes is that the lipoplexes are highly unlikely to trigger the production of neutralizing antibodies to the vector construct – making them more likely to escape any dampening effect of antibody neutralization. Our results show that the pDNA in such lipoplexes is largely unaffected by nuclease treatment [see Ref. [8]], indicating that the lipoplexed-pDNA is shielded/protected by complexation with the lipids. Of crucial importance is the fact that, unlike viral vectors which often bear immunogenic proteins on their surface (and therefore are subject to antibody neutralization and possible elimination by the immune system) [38], the lipoplexes used in the present work do not carry any protein on their surface, but instead are engrafted with a short flagellin-related peptide (9Flg). The use of such short peptide instead of an intact protein or wild-type flagellin also can be expected to reduce the risk of triggering the production of neutralizing antibody in the host. This renders DNA vaccination with 9Flg-lipoplexes a highly effective approach that is not subject to the dampening effects of antibody neutralization – an effect more

likely to be problematic when using viral DNA delivery systems [38].

In summary, we show herein for the first time that peptide ligands can be engrafted onto near-neutral lipoplexes containing NTA$_3$-DTDA to promote gene delivery to APCs *in vitro* and *in vivo*. The lipoplexes are targeted to APCs by virtue of the engrafted peptide 9Flg, which contains the flagellin-related sequence namely amino acids 85–111 of flagellin FliC. Our results show that vaccination with NTA$_3$-DTDA-containing lipoplexes engrafted with 9Flg peptide is a convenient strategy for development of effective DNA vaccines as demonstrated by the potent anti-tumour effects induced in the B16-OVA tumour model.

Acknowledgements

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