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Nanoparticle Uptake by Circulating Leukocytes: A Major Barrier to Tumor Delivery

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Abstract

Decades of research into improving drug delivery to tumors has documented uptake of particulate delivery systems by resident macrophages in the lung, liver, and spleen, and correlated short circulation times with reduced tumor accumulation. An implicit assumption in these studies is that nanoparticles present in the blood are available for distribution to the tumor. This study documents significant levels of lipoplex uptake by circulating leukocytes, and its effect on distribution to the tumor and other organs. In agreement with previous studies, PEGylation dramatically extends circulation times and enhances tumor delivery. However, our studies suggest that this relationship is not straightforward, and that particle sequestration by leukocytes can significantly alter biodistribution, especially with non-PEGylated nanoparticle formulations. We conclude that leukocyte uptake should be considered in biodistribution studies, and that delivery to these circulating cells may present opportunities for treating viral infections and leukemia.

Keywords

Lipoplexes; leukocyte uptake; gene delivery; PEGylation

Introduction

The use of nanoparticles as drug delivery vehicles offers the potential for reduced toxicity, greater efficacy, and enhanced retention at the target site. While this approach could potentially be used for delivery to any tissue, the predominant focus of current nanoparticle delivery is to achieve selective drug accumulation in tumors [1]. It is well known that nanoparticle-mediated delivery to tumors via intravenous administration is aided by the abnormal vasculature associated with rapidly growing tumors, especially in animal models [2, 3]. In spite of this advantage, the amount of the injected dose that accumulates in tumors

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remains very low ($\approx 1\%$), even in animal models [4]. It follows that the vast majority of the injected dose ($> 90\%$) accumulates in tissues other than the tumor, primarily in the lungs, liver and spleen, and the potential for toxicity of both the drug cargo and the delivery vehicle needs to be considered [5–11]. Furthermore, the resident macrophages in these organs are the major components of the reticuloendothelial system (RES) responsible for nanoparticle clearance that is thought to play the predominant role in limiting particle deposition in the tumor [12, 13]. Accordingly, strategies that reduce uptake by resident immune cells in the lung, liver, and spleen have been shown to prolong circulation times and improve delivery to the tumor [13–16]. More recently, it has been suggested that this uptake by immune cells could be exploited for immuno-oncology, and usher in a new era for cancer nanomedicine [17].

It should be recognized that intravenous administration introduces nanoparticles into the vasculature where delivery systems immediately come in contact with serum proteins and blood cells. The effects of serum proteins on the stability of delivery systems is well studied, and the tendency of researchers to optimize formulations for *in vitro* transfection under conditions of low/no serum undoubtedly contributes to the inability of many formulations to perform well *in vivo* [18–23]. In addition to the effects of serum proteins, intravenously-injected nanoparticles interact with circulating blood cells, and fusogenic particle formulations have been shown to cause aggregation of red blood cells that promotes rapid accumulation in the lung [24–27]. Some researchers have attempted to exploit interactions with red and white blood cells (“hitchhiking”) to achieve prolonged circulation and facilitate delivery to sites of inflammation and cancer [28–31]. However, other studies have demonstrated that some formulations are avidly taken up by circulating leukocytes, and shown that monocytes ultimately migrate to the RES tissues and differentiate into resident macrophages [32–37]. Therefore, initial interactions with circulating cells can potentially lead to the eventual accumulation of nanoparticles in the lung, liver, and spleen. Regardless of the specific interactions with various cell types after intravenous administration, uptake by non-target cells ultimately sequesters nanoparticles and affects drug delivery to the target tissue (e.g., tumor). Although targeting ligands can promote the retention of deposited nanoparticles in the target tissue, non-specific uptake by blood and/or immune cells nullifies targeting by preventing the ligand from gaining access to its receptor on the target tissue. It follows that strategies that reduce uptake/clearance by non-target tissues not only reduce toxicity in those tissues, but also increase the potential for systemically-administered nanoparticles to passively deposit in the target tissue.

Although interactions between delivery systems and circulating cells is well documented, strategies attempting to extend circulation times and increase delivery to the tumor predominantly focus on preventing uptake by resident macrophages in the lung, liver, and spleen. Furthermore, it is common practice to measure bioavailability by harvesting a blood sample and quantifying levels of radio-labelled nanoparticles under the assumption that circulating nanoparticles have the potential to distribute to target tissues. While blood levels are the traditional measure of “bioavailability” used for conventional small molecule pharmaceuticals, this approach ignores the potential for particles to be sequestered by circulating blood cells and thereby unavailable for diffusion into target tissues despite being present in the blood.

Our previous work has demonstrated that the serum stability of lipoplexes can be greatly enhanced by including high levels of cholesterol, consistent with earlier work with lipid-based delivery systems [23, 38–42]. We also documented that lipoplexes can be formulated to promote the formation of cholesterol domains, which have been shown to enhance serum stability and transfection rates *in vitro* and *in vivo* [40, 43–46]. More recently, we have substituted sphingosine for synthetic cationic lipids, and greatly reduced the toxicity and immunogenicity of the delivery system [5, 6, 47]. This optimized formulation was employed in the current study to investigate the uptake of lipoplexes by blood cells, and the concomitant effects on circulation times and tissue distribution. In addition, we use flow cytometry to quantify uptake by specific blood cells and probe the relationships among plasma levels, leukocyte uptake, half-life, tissue distribution, and tumor deposition.

Methods

Materials

Cholesterol, N-(1-(2, 3-dioleoyloxy) propyl)-N, N, N-trimethylammonium chloride (DOTAP), diarachidoyl-sn-glycero-3-phosphocholine (DAPC), egg phosphatidylcholine, sphingosine, rhodamine-phosphatidylethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), and PEG750-ceramide were purchased from Avanti Polar Lipids (Alabaster, AL). PicoGreen® was obtained from Thermo Fisher Scientific (Grand Island, NY), and fluorescently-labelled CD45, CD11b, CD335, CD8, and CD4 antibodies were purchased from BioLegend (San Diego, CA).

Lipoplex preparation

Lipids were dissolved in chloroform and used to prepare liposomes in water as previously described [6, 23, 43, 45]. Lipoplexes were then prepared with different lipid components and at different $\pm$ charge ratios by mixing equal volumes of a modified (CMV removed, ROSA26 added) pSelect-LucSh (Invivogen, San Diego, CA) plasmid encoding luciferase (purified and eluted in water via the “salt-sensitive” protocol (including the additional PB buffer wash, incubation of column during PE buffer wash step, elution with water, incubation of water on column during elution, and storage of eluted DNA at -20°C) in a Qiagen Maxiprep (Germantown, MD) with the suspended liposomes as previously described [6, 48]. The diameters of these preparations ranged from 190 to 310 nm as shown in our previous studies [5, 6, 49]. PicoGreen® labeling was done per manufacturer’s instructions (ThermoFisher Grand Island, NY). Briefly, in plastic-ware as recommended, a 1:10 working solution was made of the PicoGreen® dye. Forty $\mu\text{g/mL}$ DNA was incubated with the working solution for at least 10 minutes protected from light. Lipoplexes were then made with the labeled DNA. For PE-rhodamine and PE-carboxyfluorescein labeling in the flow cytometry studies, 1% of the label was added to lipoplex formulations prior to drying the lipids down.

Animal studies

For *in vivo* experiments, lipoplex preparations were concentrated by filtration as previously described [41, 45], and diluted 1:1 (v/v) with 12% hydroxyl ethyl starch (MW 250,000,

Fresenius; Linz, Austria) prior to administration. To simulate infusion conditions in the clinic, suspensions containing 50 µg DNA in 200 µl were slowly injected via tail vein (over approximately 10 seconds). We have demonstrated that this approach sharply *reduces* transfection (> 100-fold) by avoiding hydrodynamic effects that are known to enhance delivery (data not shown)[50]. Prior to treatment with lipoplexes, female immunocompetent Balb/c or SCID (NOD.CB17-Prkdc^{scid}/J) mice 6–10 weeks old were acquired from Jackson labs (Bar Harbor, ME) and inoculated in the flank with one million CT26.WT cells (murine colon carcinoma, ATCC® CRL-2638). After tumor volumes reached approximately 100 mm³ (≈ 7 days), each mouse received a single intravenous injection of lipoplexes via tail vein, and animals were sacrificed 1 h or 24 h after injection. As described previously, luciferase expression was monitored in extracted tissues with Promega Luciferase Assay Reagents (Madison, WI) [23]. All animal procedures were approved by the IACUC committee of The University of Colorado Anschutz Medical Campus, and conformed to the guidelines established by the National Institutes of Health.

Determination of plasmid levels in tissues

To determine delivery of plasmid DNA to mouse tissues, animals were sacrificed 1 h or 24 h after lipoplex administration, and extracted organs were harvested and flash frozen in liquid nitrogen. Organs were subsequently thawed, weighed, and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Quantitative PCR (qPCR) was performed on tissue samples using QuantiTech RTPCR Kit (Qiagen, Germantown, MD) on an Applied Biosystems 7500 RTPCR instrument (Grand Island, NY) as previously described [5]. A standard curve of pure plasmid was used for quantification as well as amplicon efficiency factors that account for amplification that is not perfectly efficient [5, 47, 51]. In addition, extraction efficiency was also determined for each organ and formulation, and these extraction factors were used to calculate the percent of injected dose. Although these factors that account for the inefficiencies of amplification and extraction greatly improve the accuracy of our data, variabilities in the volume injected, blood volume, and tissue weights result in errors in quantitation via PCR that are avoided in studies that employ radioactive isotopes. However, detection via PCR requires that the amplified sequence of the plasmid remain intact, and thus provides a valuable measure of integrity that is not achieved via radiolabeling.

Uptake in isolated blood

Blood harvested from tumor-bearing mice via cardiac puncture was collected into sodium citrate-containing tubes, and was immediately used to evaluate lipoplex association with the blood cell fraction. Different lipoplex formulations were incubated in freshly isolated blood for 2 h prior to centrifugation at 2000 × g to separate plasma from the cell fraction. Plasmid levels in each compartment were isolated via Qiagen kit (Qiagen, Germantown, MD) and quantified by quantitative PCR as described above. Calculation of percent injected dose assumed a blood volume of 2 mL and a 50% volume fraction of plasma.

Analyses of blood cell uptake

Blood samples (collected as described above) were subjected to eBioscience (ThermoFisher Grand Island, NY) One-Step fix/lyse procedure. Briefly, samples were aliquoted into 100 µl

and fluorescently-labelled antibodies (BioLegend San Diego, CA) were added per manufacturer's instructions. Fluorescently-labelled CD45-PE, CD11b-BV786, CD335-Alexa647, CD8-BUV395, and CD4-APCe780 antibodies were used to quantify uptake by leukocytes, myeloid cells (CD45+, CD11b+, CD335-), NK cells (CD45+, CD11b+, CD335-), CD8+ T cells (CD45+, CD8+, CD4-, CD11b-), and CD4+ T cells (CD45+, CD4+, CD8-, CD11b-). These antibodies were incubated with the samples for 60 minutes in the dark. Following incubation, 2 mL of fix/lyse solution were added to the samples and vortexed thoroughly. The samples were then incubated 60 more minutes in the dark before the cells were spun out at $500 \times g$ for 5 minutes. The pellet was then washed with 2 mL of eBioscience staining buffer (ThermoFisher Grand Island, NY). The cells were spun again at $500 \times g$ and resuspended in 200–500 μ L before they were run on the ZE5 Cell Analyzer (formerly the Propel Labs Yeti; BioRad, Hercules, CA). Spillover compensation was adjusted using Invitrogen UltraComp beads (ThermoFisher, Grand Island, NY). Samples were analyzed at a flow rate of 1 μ L/sec with an enhanced sample probe wash between samples to eliminate sample carryover. Forward light scatter measurements were collected from both the 405 nm laser and the 488 nm laser to assist in exclusion gating of free fluorescent particles. These experiments were conducted in the University of Colorado Flow Cytometry Core Facility.

Circulation times

To monitor plasmid levels in the blood, individuals were bled (15–50 μ L) at 5, 30, 60, and 120 minutes using their submandibular veins as previously described [20]. Blood samples collected at sacrifice via cardiac puncture (24 h) were also used to characterize circulation times. Blood was collected and centrifuged ($2,000 \times g$ for 10 minutes) to separate the blood cell fraction from the plasma. Each sample was then prepared for Qiagen DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). Both the cell pellet and the plasma portion were treated with the lysis buffer that comes with the kit per manufacturer's instructions. The samples were then subject to quantitative PCR. A standard curve of pure plasmid was used to determine quantity as described above. The resulting curves were fit using a first order logarithmic function within the GraphPad Prism program with R^2 ranging from 0.77–0.99.

RESULTS

Early studies with conventional, multilamellar liposomes demonstrated that leukocytes are capable of internalizing lipid-based particles [52, 53]. Uptake by leukocytes is part of the innate immune system which has evolved to provide efficient surveillance for foreign pathogens [54]. It follows that the introduction of nanoparticles via intravenous injection mimics the infection process, and thus might trigger sequestration by leukocytes. In addition, previous studies have demonstrated that intravenously-administered lipoplexes can also become associated with red blood cells that alter biodistribution [24–27]. To assess the potential for particle uptake by blood cells, lipoplexes were incubated for 2 h in blood freshly isolated from healthy mice, and we determined that the vast majority of the plasmid was associated with the blood cell fraction (Table 1). These experiments also revealed that neither changes in charge ratio (anionic or cationic) nor lipid composition could prevent the majority of lipoplexes from being associated with the blood cell fraction.

Our next set of experiments aimed to characterize the particle uptake by blood cells after intravenous administration. Because both red blood cells and leukocytes are known to interact with lipoplexes, PicoGreen®-labelled plasmid was used to prepare lipoplexes (sphingosine:cholesterol:DAPC, 3:2:5; $\pm = 0.5$; 280.9 ± 10.8 nm; -24.4 ± 2.9 mV [47]) that were injected intravenously in immunocompetent mice bearing CT26 tumors. Blood was harvested 1 hour after injection, and flow cytometry was performed on whole blood to determine the relative uptake by red and white blood cells. As shown in Figure 1A, a large fraction ($> 80\%$) of CD45⁺ cells were labelled with PicoGreen® after a single injection, indicating a strong interaction of leukocytes with these lipoplexes. In contrast, PicoGreen® labelling was barely evident in CD45-negative blood cells, suggesting that red blood cells have minimal interaction ($< 1\%$ labelled) with this lipoplex formulation. Furthermore, the PicoGreen® staining intensity of CD45⁻ cells was very low compared with that observed in CD45⁺ cells, consistent with a minimal interaction with red blood cells and a strong interaction with leukocytes (Fig. 1B+C). Moreover, the stark differences in staining suggest that the interaction is not simply due to random collisions with circulating cells, but that leukocytes actively uptake lipoplexes in the blood.

Additional experiments were conducted to determine whether specific leukocyte populations were responsible for lipoplex uptake. The data in Figure 2 indicate that almost all myeloid cells had taken up lipoplexes within one hour, and NK cells also exhibited similar uptake ($\approx 99\%$ labelled). In comparison, 58% of CD8⁺ T cells and 40% CD4⁺ T cells had taken up lipoplexes. Consistent with the suggestion that the interaction between blood cells and lipoplexes is not merely due to random association/collision, the different levels of interaction observed by the various types of leukocytes suggest specific/active uptake.

In an attempt to understand the relationship between blood cell uptake and tissue distribution, blood and tissues were harvested from tumor-bearing mice 1 h after injection, and deposition in each tissue is expressed as the percent of the injected dose (% ID). At this timepoint, approximately 25% of the injected plasmid was present in the blood, and the vast majority of that was associated with the cell fraction (Fig. 3A). Considering the high leukocyte uptake noted above combined with the low levels in the plasma, it is not surprising that tumor accumulation was minimal ($\approx 0.04\%$ ID) after 1 h (Fig. 3B). Furthermore, plasmid levels in the tumor were reduced after 24 h, consistent with the low level of expression observed (Fig. 3B+C). Plasmid levels in other tissues were also quantified, and significant amounts were observed in all organs 1 h after injection, with sharp reductions after 24 h (Fig. 4A). Expression (24 h) was highest in liver and lung despite greater plasmid levels in the spleen and kidney (Fig. 4B).

It is well known that coating particles with polyethyleneglycol (PEG) reduces uptake by phagocytic cells, and this correlates with increased delivery to tumors [16, 36, 55, 56]. To test the effect of PEG on leukocyte uptake, PEGylated lipoplexes (5% PEG750-ceramide) were administered to tumor-bearing mice and blood was analyzed after 1 and 24 h. PEGylation dramatically reduced overall leukocyte uptake to $< 30\%$ labelled (CD45⁺; compare figs. 1A to 5A), and this reduced uptake was especially evident in CD8⁺ and CD4⁺ T cells (Fig. 5B). In sharp contrast to that observed with non-PEGylated lipoplexes, 77% of the injected dose was free in the plasma after 1 h, and only 3% associated with the blood cell

fraction (Fig. 6A). The reduced leukocyte uptake correlated with enhanced delivery to the tumor (0.82% ID); approximately 23-fold greater than that observed for non-PEGylated lipoplexes at 1 h (Fig. 6B). In sharp contrast to non-PEGylated lipoplexes, tumor accumulation was dramatically increased after 24 h (2.35% ID), suggesting additional deposition of free lipoplexes circulating in the plasma. However, accumulation of lipoplexes in the liver was also increased dramatically one hour after injection (compare figs. 4A + 7A). Interestingly, the levels of PEGylated lipoplexes in the lung decreased at 24 h, whereas levels in the liver and spleen increased dramatically. These results are consistent with previous studies reporting that lipoplexes can initially become entrapped in the lung, but ultimately re-enter the circulation and are cleared by the liver and spleen [24, 41]. Expression at 24 h was markedly increased in all tissues (except kidney) as compared to non-PEGylated formulations, consistent with the greater plasmid levels observed at this timepoint (compare figs. 4B and 7B).

To further assess the relationship between leukocyte uptake and tissue delivery, tumor-bearing SCID mice characterized by markedly lower amounts of circulating leukocytes (410 vs. 3240 cells/ μ l; Mouse Phenome Database, www.jax.org/phenome) were employed in a series of experiments. Because the circulating leukocyte levels in SCID mice are approximately 13% of normal Balb/c mice, it would be expected that lipoplexes might have greater potential for deposition in the tumor and other organs. Accordingly, parallel experiments with non-PEGylated lipoplexes were performed on tumor-bearing SCID mice. As shown in Figure 8A, delivery to the tumor was similar to PEGylated formulations at 1 h (0.80 ± 0.10 % ID), and slightly higher at 24 h (2.77 ± 0.13 % ID). Major accumulation was observed in the liver where high levels remained at 24 h. Expression in organs was comparable to normal mice administered PEGylated lipoplexes, with the exception of dramatic reduction in the spleen (Fig. 8B).

Additional measurements were conducted to compare the circulation times of non-PEGylated and PEGylated lipoplexes in normal mice, as well as non-PEGylated lipoplexes in SCID mice. Because of the dramatic differences in leukocyte accumulation, lipoplex levels in the plasma and blood cell fraction were quantified separately. In sharp contrast to non-PEGylated lipoplexes, PEGylated lipoplexes exhibited prolonged circulation in the plasma with minimal levels in the blood cell fraction (Fig. 9). Plasma levels of non-PEGylated lipoplexes in SCID mice were approximately two-fold greater than normal mice, but still well below that seen with PEGylated lipoplexes. The half-lives of lipoplexes in the plasma, blood cell fraction and total blood are shown in Table 2.

DISCUSSION

Researchers have been attempting to improve delivery to tumors for decades [14–16]. It was recognized in early studies with liposomes that resident macrophages in the lung, liver, and spleen are part of the endogenous system of clearing foreign particulates, and studies have predominantly focused on reducing uptake in these organs [15, 16, 57, 58]. Although some studies have demonstrated the ability of monocytes and neutrophils to phagocytose nanoparticles [29–32, 35, 36], the potential for uptake by circulating leukocytes is generally not considered [33]. This issue is complicated by the migration of some leukocytes (e.g.,

monocytes) to tissues and their subsequent differentiation into resident immune cells [32, 33]. Therefore, migration of nanoparticle-containing leukocytes to the lung, liver, and spleen could potentially contribute to the accumulation observed in resident macrophages that are implicated in clearance.

Although our study did not investigate the ultimate fate of phagocytosing cells, we have observed that a surprisingly large portion (87% ID) of our non-PEGylated lipoplex formulation is associated with the blood cell fraction 5 min after intravenous injection, and this rapidly drops to 22% after 1 h (Fig. 9A). Analysis of uptake of fluorescently-labelled lipoplexes by blood cells 1 h after injection indicates that the vast majority of leukocytes exhibit high levels of fluorescence, in contrast to minimal labeling of erythrocytes (Fig. 1). The specific labelling of leukocytes, as compared to red blood cells, indicates that uptake occurs via active phagocytosis as opposed to an indiscriminant collision of fusogenic lipoplexes with blood cells [52, 53]. Additional analyses revealed that almost all myeloid and NK cells are labelled after a single intravenous injection, and that CD8+ and CD4+ T cells also exhibit substantial particle uptake (Fig. 2). This high level of uptake by leukocytes corresponded with relatively low accumulation of lipoplexes in the tumor and other organs (Figs. 3 + 4). Consistent with clearance of circulating leukocytes that have internalized lipoplexes, the highest plasmid levels at 24 h are observed in the spleen (Fig. 4A). This is consistent with the reduced levels of leukocytes containing fluorescent labelling after 24 h (Figs 2 + 5B), and the short lifetimes (10–20 h) of neutrophils and monocytes [31]. It is also likely that internalized plasmid is degraded within leukocytes between 1 and 24 h. Taken together, these findings demonstrate that leukocyte uptake can play a significant role in the distribution and clearance of intravenously-injected nanoparticles. While uptake by red blood cells was minimal as compared to leukocytes (Fig. 1), it is important to recognize that erythrocytes are > 1000-fold more numerous (Mouse Phenome Database, www.jax.org/phenome), and thus even much weaker interactions with red blood cells could significantly affect distribution.

In contrast to the uncoated lipoplexes used in experiments depicted in Figs. 1–4, contemporary nanoparticles typically employ some type of polymeric coating (e.g., PEG, poloxamer, polyethylenoxide, dextran) to extend circulation times and improve biodistribution [32, 33, 35, 36]. Of these coating strategies, PEGylation is by far the most common despite the expanding number of studies documenting problems associated with its immunogenicity [47, 59–63]. Consistent with the ability of PEG to prevent uptake by macrophages, leukocyte uptake was dramatically reduced (29% vs. 87% particle positive), and the vast majority of circulating lipoplexes were present in the plasma (Figs. 6A, 9B). The decreased leukocyte uptake and higher plasma levels corresponded with increased delivery to the tumor (Figs. 6B vs. 3B). However, it is important to note that delivery to other organs was also increased dramatically, suggesting that the reduced sequestration by leukocytes does not cause preferential accumulation in the tumor, but provides the opportunity for further particle deposition in all tissues (Fig. 7). At 1 h, we observed high levels of PEGylated lipoplexes in the liver and lungs, but lung accumulation is reduced at 24 h as opposed to significant increases in liver and spleen (Fig. 7A). It is worth noting that all of the injected dose of plasmid incorporated in PEGylated lipoplexes can be accounted for by that accumulated in organs at 24 h. In contrast, < 10% of plasmid injected in non-

PEGylated lipoplexes is present in organs at 24 h. This difference could potentially indicate that few of the leukocytes involved in uptake of non-PEGylated formulations migrate to organs and become resident immune cells. However, we feel that it is more likely that leukocyte sequestration may facilitate elimination and/or degradation that prevents tissue accumulation and/or detection via PCR.

In a tumor-bearing SCID mouse model possessing dramatically lower levels of circulating leukocytes, higher levels of injected lipoplexes (non-PEGylated) were found in the plasma at 1 h (as compared to the same formulation administered to normal mice; Figs. 9A vs 9C), and this corresponded with greater deposition in tumor and liver at this timepoint (Figs. 3B, 4A, 8). Although the plasma levels are greater in SCID mice as compared to normal mice, the blood cell fraction still possessed a large portion of the injected plasmid at early timepoints (Fig. 9C). In sharp contrast to that observed in normal mice, this plasmid appears to accumulate in tissues at 24 h, suggesting that the normal process of leukocyte-mediated degradation/clearance is altered in SCID mice. We observed a similar effect with PEGylated lipoplexes in normal mice, i.e., organ accumulation at 24 h could account for all of the injected dose. Considering that phagocytosis of foreign material by leukocytes triggers apoptosis in order to minimize inflammation and promote rapid resolution of infections, it would be expected that the uptake of lipoplexes we observe would result in abrupt degradation of the plasmid and clearance of associated leukocytes [64, 65]. Indeed, our *inability* to detect a significant fraction of the plasmid when normal mice are injected with non-PEGylated lipoplexes is consistent with this scenario. However, the ability to account for the injected plasmid (even at 24 h) in experiments involving both the SCID phenotype and PEGylation, indicates that normal degradation/clearance is altered under both these conditions. Given the severe immunodeficiency associated with the SCID phenotype, it is understood that the normal function of the innate immune system is severely compromised in these mice. In the case of PEGylation, previous studies have suggested that regular processing via the endosomal/lysosomal pathway is attenuated [66, 67]. It is also worth noting that inflammatory cytokines can postpone leukocyte apoptosis and elimination [64, 65]. More specifically, G-CSF, GM-CSF, IL-1 β , IL-6, and IFN- γ have each been shown to prolong leukocyte lifetimes [65], and we have documented that all of these cytokines are produced when this specific lipoplex formulation is PEGylated [47]. Ironically, the lack of cytokine response associated with the non-PEGylated formulation (something we have strived to achieve) may contribute to its rapid degradation/clearance and might ultimately be responsible for our inability to account for much of the injected dose. However, the rapid clearance associated with the lack of a cytokine response may be beneficial for reducing adverse effects.

As described above, both PEGylation and the use of SCID mice increased plasma levels and extended blood half-life (Fig. 9, Table 2). While the effect of SCID mice on plasma levels was small relative to that seen with PEGylation, plasmid accumulation in the tumor was greater in SCID mice despite a shorter circulation half-life (Table 2). Although we observe progressive tumor accumulation with the PEGylated formulation (i.e., 24 h > 1 h), delivery to the tumor was greater in SCID mice administered non-PEGylated lipoplexes despite the longer plasma half-life of PEGylated lipoplexes. Curiously, the greater plasmid levels

observed in the tumors of SCID mice resulted in enhanced expression as compared to normal mice, but lower expression than that seen with PEGylated lipoplexes.

In addition to the poor correlation with plasma half-life, we also do not observe any consistent correlation of delivery with plasmid levels in the blood cell fraction. The severely compromised immune system (in SCID mice) and the potential for cytokines to alter leukocyte-mediated degradation (discussed above) likely contribute to the inability to use plasmid levels in the blood (plasma or cell fraction) as a reliable predictor of tumor delivery. This exposes a potential complication with using methods that do not account for particle sequestration by blood cells, i.e., nanoparticles within leukocytes can represent a large portion of the total blood levels even though such particles are essentially already “cleared” within circulating leukocytes and thereby unavailable for direct deposition into target tissues. We suspect that this is especially relevant to nanoparticle formulations that activate complement and are readily phagocytosed by circulating leukocytes [68]. While radiolabeling allows total blood levels to be readily determined with a scintillation counter, our results suggest that separate quantification of the plasma and cellular components can provide useful insight into pharmacokinetics and its relation to drug distribution. It is worth noting that some previous studies that intentionally couple enzymes to erythrocytes have conducted such measurements, but this is atypical [69].

In conclusion, this study demonstrates that particulate formulations can be readily phagocytosed by circulating leukocytes in the blood. It follows that the potential for leukocyte sequestration alters the conventional relationship between circulating drug levels and “bioavailability”. We suggest that circulating leukocytes represent another “compartment” that should be considered when investigating nanoparticle formulations. Our study focuses on achieving effective delivery to the tumor, and we demonstrate that sequestration within leukocytes and the associated clearance can present a significant barrier to tumor delivery. Therefore, the ability of PEG to dramatically reduce leukocyte uptake appears to play a predominant role in its well established ability to increase accumulation in the tumor and other tissues. Similarly, the ability to avoid leukocyte uptake may explain the reduced incidence of neutropenia in patients administered PEGylated (Doxil®) as compared to non-PEGylated (Myocet®) liposomal doxorubicin [70]. It is important to note that many viruses rely on leukocyte uptake to disseminate particles during infection, and thus delivery to leukocytes might represent an effective strategy for combating infection and distributing therapeutics to infected organs. According to this scenario, the administration of the lipoplexes used in this study (and presumably other nanoparticles) simulates an infection that elicits leukocyte uptake that is characteristic of an innate immune response. In addition, uptake by leukocytes could prove advantageous for treating leukemia, and might also provide novel avenues for effective leukocyte labelling *in vivo* that could allow for improved imaging of inflamed tissues [34].

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Figure 1.

Analysis of lipoplex uptake by blood cells via flow cytometry. Balb/c mice ($n = 3$) bearing CT26 tumors were intravenously injected with PicoGreen®-labelled lipoplexes, and blood was harvested after 1 h. Blood cells were stained with an antibody against CD45 which binds to leukocytes. The percentage of CD45- and CD45+ cells that are positive for PicoGreen® staining (A), and the staining intensity of CD45+ (B) and CD45- (C) are depicted. Note that PicoGreen® staining is largely confined to leukocytes (CD45+), with minimal staining of erythrocytes (CD45-). In addition, the intensity of PicoGreen® staining is much higher in leukocytes.

Figure 2.

Analysis of lipoplex uptake by leukocytes via flow cytometry. Balb/c mice bearing CT26 tumors were intravenously injected with carboxyfluorescein-labelled lipoplexes, and blood was harvested after 1 h. CD45⁺ leukocytes were stained with antibodies to quantify uptake by myeloid cells (CD45⁺, CD11b⁺, CD335⁻), NK cells (CD45⁺, CD11b⁺, CD335⁺), CD8⁺ T cells (CD45⁺, CD8⁺, CD4⁻, CD11b⁻), and CD4⁺ T cells (CD45⁺, CD4⁺, CD8⁻, CD11b⁻). The percent of each cell population that was particle positive is depicted. Bars represent the mean and standard error of blood isolated from 3 mice.

Figure 3.

Time dependence of lipoplex distribution. Plasmid levels in both the plasma and the cell fraction of the blood (A) and corresponding levels in the tumor (B) are shown at 1 and 24 h. Consistent with the low levels of plasmid, expression (24 h) in the tumor was minimal (C). Bars represent the mean and standard error of samples harvested from 3 mice at each timepoint.

Figure 4. Biodistribution and expression in organs. Plasmid levels decreased in all organs between 1 and 24 h (A). Expression at 24 h (B) in organs does not correlate with plasmid levels. Bars represent the mean and standard error of organs harvested from 3 mice at each timepoint.

Figure 5.

Leukocyte uptake of PEGylated lipoplexes. Uptake of PEGylated lipoplexes by CD45+ cells (A) decreases between 1 and 24 h. Uptake of PEGylated lipoplexes by specific cell types at 1 and 24 h (B): myeloid cells (CD45+, CD11b+, CD335-), NK cells (CD45+, CD11b+, CD335+), CD8+ T cells (CD45+, CD8+, CD4-, CD11b-), and CD4+ T cells (CD45+, CD4+, CD8-, CD11b-). The percent of each cell population that was particle positive is depicted. Bars represent the mean and standard error of blood isolated from 3 mice.

Figure 6. Time dependence of PEGylated lipoplex distribution. Plasmid levels in the plasma were much higher than the cell fraction (A), and corresponded with higher tumor levels (B) as compared to non-PEGylated lipoplexes. Note that plasmid levels in the tumor increase from 1 h to 24 h, consistent with the longer half-life (≈ 3 h) of the PEGylated formulation. Bars represent the mean and standard error of tissues harvested from 3 mice at each timepoint.

Figure 7. Biodistribution of PEGylated lipoplexes. Plasmid levels increased at 24 h in both liver and spleen (A). Expression (24 h) was highest in liver and spleen, consistent with the greater plasmid accumulation (B). Bars represent the mean and standard error of organs harvested from 3 mice at each timepoint.

Figure 8.

Biodistribution of lipoplex formulations in SCID mice. Plasmid accumulation was greatest in the liver, and accumulation in tumor, lung, and spleen were comparable (A). Expression (24 h) was highest in liver, consistent with the greater plasmid accumulation (B). Bars represent the mean and standard error of organs harvested from 3 mice at each timepoint.

Figure 9.

Blood circulation. Plasmid levels in the plasma and cell fraction were quantified separately at different time points for non-PEGylated (A) and PEGylated (B) lipoplexes in Balb/c mice, and non-PEGylated lipoplexes in SCID mice (C). Note the much greater levels of PEGylated lipoplexes in the plasma; calculated half-lives are presented in Table 2. Each symbol represents the mean and standard error of blood samples from 3 mice.

Table 1.

Effect of lipoplex formulation on association with the blood cell fraction after a 2 h incubation in blood freshly isolated from Balb/c mice. n = 3.

Formulation	Charge Ratio (+/-)	% in Cell Fraction
Sphingosine:cholesterol:DAPC 3:2:5	0.5	89.35 ± 8.91
Sphingosine:cholesterol:DAPC 3:2:5	4	79.10 ± 11.48
Sphingosine:cholesterol 1:4	0.5	84.49 ± 9.07
Sphingosine:cholesterol 1:4	4	95.35 ± 10.61
DOTAP:cholesterol 1:4	0.5	91.35 ± 12.04
DOTAP:cholesterol 1:4	4	93.46 ± 12.82
DOTAP:cholesterol 1:1	0.5	53.46 ± 15.55
DOTAP:cholesterol 1:1	4	69.03 ± 9.23

Table 2.

Half-lives (in minutes) of lipoplexes in different blood fractions calculated from the plasmid levels depicted in Figure 9.

	Plasma	Cell Fraction	Whole Blood
Balb/c	32.33 ± 0.01	30.95 ± 5.70	31.10 ± 1.41
SCID	61.56 ± 2.71	33.51 ± 3.97	56.17 ± 1.10
PEG	186.5 ± 1.25	19.81 ± 13.85	151.30 ± 1.30