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Hypersensitivity and Loss of Disease Site Targeting Caused by Antibody Responses to PEGylated Liposomes

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The systemic application of nucleic acid drugs requires delivery systems that overcome the poor pharmacokinetics, limited biodistribution, and inefficient uptake of nucleic acids. PEGylated liposomes show considerable promise because of their intrinsic ability to accumulate at disease sites and facilitate transfection of target cells. Unlike many viral vectors, PEGylated liposomes are generally considered to be nonimmunogenic. We have developed a PEGylated liposome for the systemic administration of plasmid DNA that achieves high levels of selective gene expression at distal tumor sites. Here we report that the *in vivo* efficacy and safety of these systems can be severely compromised following repeat administration. This phenomenon is characterized by a loss of disease site targeting, accelerated clearance from the blood, and acute hypersensitivity. These effects are fully attributable to a surprisingly robust, long-lived antibody response generated against polyethylene glycol (PEG) that results from the strong adjuvant effect of the plasmid payload. Importantly, immunogenicity may be substantially reduced by modifying the alkyl chain of the PEG–lipid conjugate, thereby allowing successful repeat dosing of the modified plasmid formulations without adverse side effects. Immunogenicity is a relevant concern for a number of nonviral delivery systems given the potent immunostimulatory properties of many nucleic acid drugs.

Key Words: non-viral vectors, immunogenicity, nucleic acid-based drugs, liposomes, antibody responses, polyethylene glycol, hypersensitive reactions

INTRODUCTION

Liposomes are an attractive drug delivery system for a diverse array of therapeutic agents due to their relatively high stability in the blood and intrinsic ability to extravasate into tissues with increased vascular permeability, such as solid tumors and sites of inflammation [1,2]. This so-called "passive disease site targeting" can be facilitated by the incorporation of polyethylene glycol (PEG) into liposomes to provide a steric barrier against opsonization and clearance by the reticuloendothelial system (RES) [3,4]. These attributes have been exploited in the field of oncology by a number of liposomal chemotherapeutic [2,5] and scintigraphic agents [1]. PEGylated liposomes also show significant potential for developing nucleic acids as therapeutic agents, particularly in applications requiring systemic administration. Lipid encapsulation of RNA or DNA provides protection from intravascular nuclease degradation, passive targeting to disease sites and can enhance the intracellular delivery of nucleic acids that are otherwise poorly taken up by cells [6].

Administration of many nucleic acids can cause activation of the mammalian immune system, leading to the release of interferons and proinflammatory cytokines. In the case of DNA, immune stimulation is triggered primarily by the recognition of unmethylated CpG sequence motifs by Toll-like receptor-9 (TLR9) [7] located within the endosomal compartment of antigen-presenting cells (APC), including B cells [8,9]. Similar immune recognition pathways are also activated by exogenous single [10,11] and double-stranded RNA [12] through TLR7/8 and TLR3, respectively. In this context, we [13] and others [14,15] have recently reported that synthetic siRNA, under development as a therapeutic mediator of RNA interference, can also induce potent immune stimulation. These immune responses elicited by nucleic acids can be greatly potentiated by the use of delivery vehicles that facilitate cellular uptake [13,16]. Although the immunomodulatory effects of CpG DNA are now being harnessed therapeutically in oncology and allergy applications [17], in many cases immune activation represents an additional hurdle to drug development

due to the significant toxicities associated with excessive cytokine release and the potential for the drug carrier to be rendered immunogenic.

It has long been recognized that liposomes can act as immunological adjuvants as a result of their particulate nature, efficient uptake by APC, and ability to crosslink surface receptors [18], and this property is enhanced when immunostimulatory agents such as CpG DNA are incorporated into the liposomes [19,20]. This has been exploited in the design of liposomal vaccines that generate strong antibody (Ab) responses against weakly immunogenic antigens grafted onto the liposome surface. It is therefore unsurprising that immunogenicity has proven to be a major obstacle in developing receptor-targeted liposomes that incorporate antibodies, peptides, or receptor ligands on their surface to enhance target cell uptake [21–23]. The addition of a PEG coating to these liposomes typically has a minor effect on reducing their immunogenicity [19,21,23].

We have developed stable plasmid lipid particles (SPLP) as a nonviral systemic vector for the expression of therapeutic pDNA at disease sites such as tumors and sites of inflammation [24,25]. SPLP consist of a PEGylated liposome that fully encapsulates a single copy of plasmid DNA, thereby conferring protection from nuclease degradation and extended blood circulation times following systemic administration [24,25]. Here we report that the *in vivo* efficacy and safety of these systems can be severely compromised following repeat administration due to a surprisingly robust Ab response against PEG that arises from the primary administration. Importantly, the immunogenicity of the PEGylated liposomes can be significantly reduced by modification of the PEG–lipid component, allowing for the safe and effective re-administration of the formulated pDNA. Our findings raise important concerns regarding the potential immunogenicity of delivery vehicles currently under consideration for use with immunostimulatory nucleic acid-based drugs, including pDNA, siRNA, and antisense oligodeoxynucleotides.

Results and Discussion

Disease Site Targeting and Blood Clearance of PEGylated Liposomes

To demonstrate the utility of these systems for the delivery of nucleic acids to distal tumor sites, we encapsulated a CMV–luciferase reporter plasmid into SPLP containing 10 mol% PEG-S-DSG (Luc-SPLP). A single intravenous (iv) administration of Luc-SPLP (5 mg/kg pDNA) into A/J mice bearing subcutaneous Neuro2a tumors on the hind flank resulted in significant reporter gene expression at the distal tumor site 48 h after administration (Fig. 1A). Transgene expression within other, nontarget organs including the liver, lungs, spleen, kidney, and heart was low (Fig. 1A). We obtained similar results in a CT26 liver metastases model in Balb/C mice (not shown).

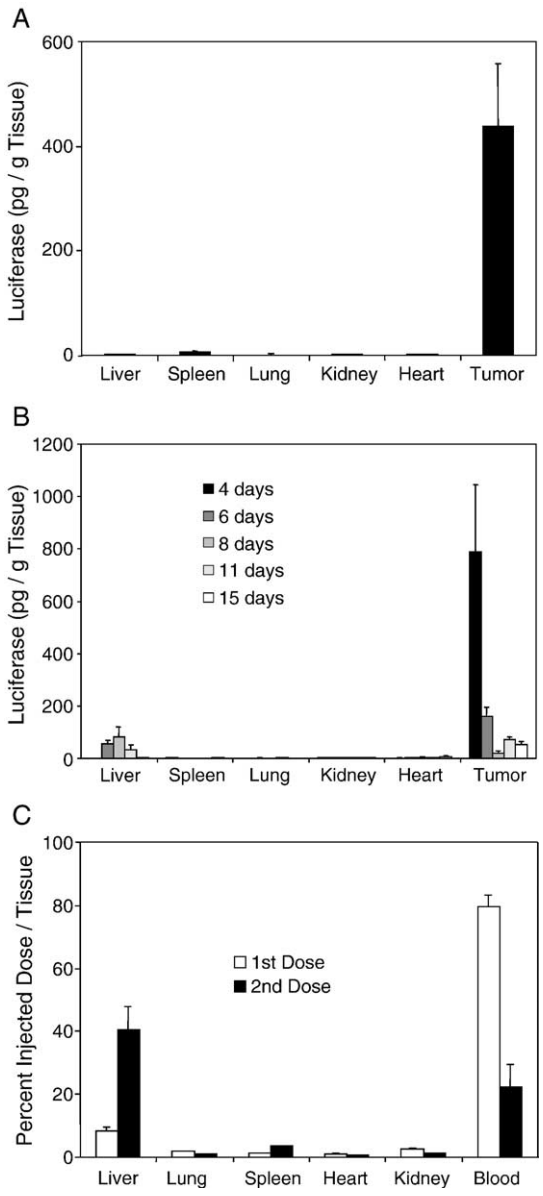

FIG. 1. Repeat administration of PEGylated liposomes is associated with loss of tumor-targeted transgene expression and accelerated blood clearance. (A) Luciferase expression in the tumor and nontarget tissues 48 h after a single iv administration of Luc-SPLP (100 μg pDNA) containing PEG-S-DSG in Neuro2a tumor-bearing A/J mice. Transgene expression is expressed as pg luciferase/g tissue. Data represent means ± SD; n = 5 mice. (B) Luciferase expression following repeat administration of Luc-SPLP. Mice were treated with Luc-SPLP 4 to 15 days prior to a second treatment with Luc-SPLP (see key for treatment intervals). Luc expression was determined 48 h after second treatment. Data are presented as in (A). (C) Biodistribution of SPLP 1 h after first or second iv administration. ICR mice were treated with unlabeled SPLP containing 100 μg pDNA. 7 days later, naïve (first dose) or pretreated animals (second dose) received ^{3}H-labeled SPLP (100 μg pDNA). Blood and major tissues were collected 1 h after radiolabeled SPLP administration and specific activity was determined. Values are expressed as percentage of injected dose/tissue (means + SD; n = 4 mice).

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To examine whether tumor-selective transgene expression was maintained following multiple treatments, we subjected mice to a subsequent administration of Luc-SPLP at increasing time intervals after the initial treatment. A second administration of Luc-SPLP given 4 days after the first resulted in Luc expression within the tumor that was comparable to that observed after a single treatment (Fig. 1B). However, when the interval between injections was extended to 6 days or greater, tumor transfection resulting from the second SPLP treatment was significantly reduced. Loss of transgene expression in the tumor after multidosing was accompanied by increased expression in the liver, suggesting that the pharmacokinetic or biodistribution profile of the second SPLP dose was adversely affected (Fig. 1B). Changes in tumor growth rate or other tumor-specific changes induced by the first SPLP treatment were unlikely to be the cause of reduced transgene expression since expression was also attenuated following injection intervals of 11 days or greater, which required initial SPLP administration to occur prior to tumor seeding. SPLP treatment had no effect on tumor growth rates (data not shown).

To determine if the dramatic change in gene expression profiles was caused by altered blood clearance or biodistribution of the second SPLP treatment, we incorporated a nonexchangeable radiolabeled lipid marker [26] into SPLP. One hour after a single iv injection of radiolabeled SPLP 80% of the injected dose remained within the blood of naive mice (Fig. 1C), consistent with an expected blood circulation half-life of 12–14 h for liposomes containing PEG-C18 lipids such as PEG-S-DSG [24,25]. Less than 10% of the labeled liposomes had accumulated in the livers of these animals over the first hour after administration (Fig. 1C). By contrast, in mice treated with SPLP 7 days earlier, only 25% of the second SPLP dose remained in the blood 1 h after iv administration. This was accompanied by significant accumulation of SPLP in the liver and, to a lesser extent, the spleen, implying involvement of the RES in the accelerated blood clearance (Fig. 1C). These results indicate that changes in gene expression pattern after multiple SPLP administrations are likely attributable to rapid elimination of the PEGylated liposomes from the blood and their redistribution to the liver.

Anti-PEG Antibodies Can Be Generated in Response to PEGylated Liposomes

The loss of tumor targeting and altered biodistribution in multidose studies with SPLP suggested an immune-mediated clearance mechanism. To examine this possibility, we developed a modified ELISA [27] to detect antibodies

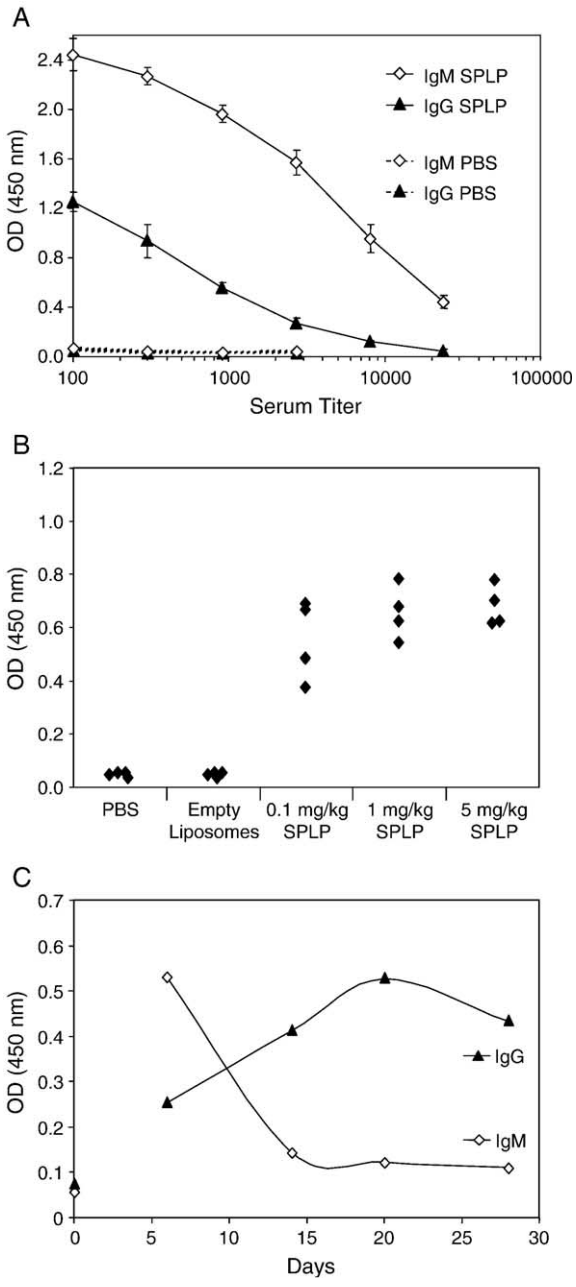

FIG. 2. PEGylated liposomes can induce long-lived antibody responses against PEG. (A) DSG-SPLP induces IgM and IgG antibodies reactive against PEG-S-DSG. ICR mice were treated with either PBS (vehicle control; dashed lines) or DSG-SPLP containing 100 μg pDNA (solid lines). Serum IgM and IgG antibodies reactive against PEG-S-DSG (anti-PEG Ab) were measured 7 days later by a modified ELISA. Pooled serum (n = 4 mice) was assayed in duplicate by serial dilution. Values represent mean ODs of three individual assays ± SD. Similar results were obtained in C57BL/6J and C3H/HeN mice. (B) Anti-PEG Ab responses are induced by low doses of SPLP. Mice were treated with SPLP at 100, 20, or 2 μg pDNA (1500–30 μg total lipid, 600–12 μg PEG-S-DSG) or empty liposomes of identical composition (equivalent to SPLP dose of 100 μg pDNA). Total anti-PEG IgG (H+L) levels were assessed in serum 7 days later. Data are OD values from serum diluted 1:100 from individual animals in each group. (C) Duration of the anti-PEG IgM and IgG response. ICR mice were treated with SPLP (100 μg) on day 0. Mice were test bled at the indicated times after treatment up to day 28. Anti-PEG IgM and IgG levels are expressed as mean OD values from pooled serum samples (n = 4) diluted 1:100 at each time point.

against the lipid components of PEGylated liposomes. Seven days after a single iv injection of SPLP we detected significant levels of IgM and IgG reactive against the PEG-S-DSG component of SPLP (anti-PEG Ab) in the serum of treated mice (Fig. 2A). These Ab were reactive against other PEG-conjugated lipids and nonreactive against the native, unconjugated lipid, suggesting that the antigenic epitope was the PEG moiety itself rather than the lipid anchor. We did not detect increased Ab reactivities against the other three lipid components of SPLP (not shown). Anti-PEG Ab were generated by relatively low doses of liposomal pDNA. Mice treated with 0.1 mg/kg SPLP (approximately 2 μg pDNA, 30 μg total lipid, 12 μg PEG-S-DSG) developed significant levels of anti-PEG Ab 7 days after administration (Fig. 2B). However, treatment with empty liposomes demonstrated that the generation of anti-PEG Ab to SPLP was entirely dependent on the encapsulated pDNA within the liposome (Fig. 2B). We determined serum levels of anti-PEG IgM and IgG over 4 weeks after a single SPLP treatment. Anti-PEG IgM levels peaked 7 days after SPLP administration and then declined rapidly to reach near pretreatment levels by day 14 (Fig. 2C). In contrast, anti-PEG IgG increased for 20 days after treatment and remained elevated through day 28.

These data demonstrate that the encapsulation of immunostimulatory pDNA within SPLP is sufficient to render the PEGylated delivery vehicle immunogenic. This manifests as a surprisingly robust, long-lived humoral immune response to PEG that is sufficient to cause accelerated blood clearance and loss of disease site targeting upon subsequent re-administration. Liposomes incorporating immunostimulatory molecules are known to act as potent adjuvants that can promote Ab responses against weakly immunogenic antigens [19,20], including lipids [27,28], displayed on the outer surface of the liposome. Reports have also shown that Ab responses against PEG can be raised when PEGylated proteins are used in conjunction with aggressive immunization regimens [29,30]. Therefore, despite PEG being typically regarded as nonimmunogenic, it is clear from the current study, as implied in the recent report by Semple *et al.* [31], that PEG itself can act as an antigenic epitope in a drug formulation when presented in the context of a strong adjuvant such as a liposome containing an immunostimulatory payload.

Since pDNA can act as a polyclonal B cell activator [7–9], we examined the B cell proliferative response to SPLP *in vivo* to determine if the production of anti-PEG Ab is part of a generalized polyclonal Ab response. When we assessed proliferation by flow-cytometric analysis of bromodeoxyuridine (BrdU) incorporation [32], we observed only a small increase in the proportion of IgM^+ B cells proliferating in the spleen following SPLP administration (Fig. 3A) compared to cells recovered from PBS (Fig. 3B) or empty liposome-treated control mice (Fig. 3C). Within the IgM^+ population, however, a distinct subset of plasmablasts was expanded specifically in SPLP-treated mice. These cells represented up to 3% of the total IgM^+ population and were defined by their expression of the plasma cell marker syndecan-1 [33] and incorporation of BrdU over the 3 days following SPLP treatment (Fig. 3A). The majority of plasmablasts exhibited reduced expression of the B cell marker B220 (not shown).

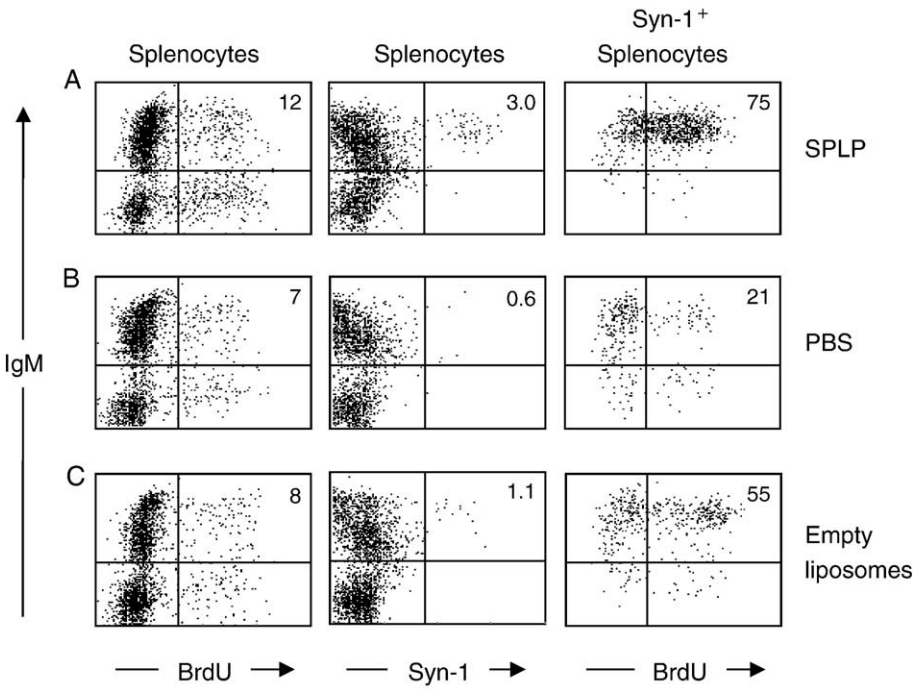

FIG. 3. B cell proliferation and differentiation in response to PEGylated liposomes. Mice were treated with (A) SPLP, (B) PBS, or (C) empty PEGylated liposomes and fed drinking water containing bromodeoxyuridine (BrdU) continually for 3 days to label proliferating cells. Four-color staining of spleen cell suspensions for flow cytometry identified IgM^+ B cells incorporating BrdU (left) or expressing the plasma cell marker syndecan-1 (Syn-1; middle) and gated $Syn\text{-}1^+$, IgM^+ cells that had incorporated BrdU (right). The majority of IgM^+, $Syn\text{-}1^+$, $BrdU^+$ plasmablasts in SPLP-treated animals had downregulated the B cell marker B220 (not shown). Plots are representative of spleens from three to six mice in each group assayed in two separate experiments. Values in upper right quadrant represent percentage IgM^+ splenocytes.

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Analysis of the early B cell response indicates that the generation of Abs against PEG likely reflects the selective activation and differentiation of a small subset of B cells rather than polyclonal B cell activation triggered by the nonspecific uptake of pDNA. We therefore envisage a multistep model leading to the production of anti-PEG Ab: first, liposome binding and crosslinking of surface immunoglobulin (sIg) on PEG-reactive B cells; second, internalization of the payload and activation of B-cell-stimulatory pathways such as TLR9 by the pDNA [9]; and third, the induction of cytokines from accessory cells that support maturation of the nascent Ab response. These signals have been shown to act in concert on B cells to generate Ab responses that are independent of T cell help [9,34,35]. Although not observed with our formulations, we do not exclude the possibility that PEGylated liposomes containing nonstimulatory payloads may also be weakly immunogenic. This may become apparent if the liposomes can more effectively crosslink sIg on B cells, a scenario analogous to thymus-independent type II Ab responses characterized by polymeric antigens with multiple repeating epitopes [36]. The efficiency of sIg crosslinking will likely be influenced by liposome size, composition, bilayer fluidity, and epitope density on the liposomal surface. Several groups have demonstrated the accelerated blood clearance of empty PEGylated liposomes in both rodents [37–40] and primates [37] following repeated administration. These phenomena have been attributed to unidentified soluble serum factors [37,40] and enhanced phagocytic activity of the RES [38]. However, it would be interesting to reexamine such models using appropriate assays to determine if Ab responses against the PEG or lipid components may be responsible.

Anti-PEG Antibodies Trigger Platelet-Activating Factor (PAF)-Dependent Hypersensitive Reactions

A single treatment with SPLP containing PEG-S-DSG was sufficient to induce hypersensitivity in mice that manifested as acute toxicity upon subsequent treatment. Symptoms typically developed 5–10 min after re-administration and included lethargy, facial puffing, vasodilation, labored respiration, and significant mortality rates at higher challenge doses. These symptoms appeared typical of an Ab-mediated anaphylactic reaction [41]. Establishment of hypersensitivity required dosing intervals of at least 6 days and was achieved at SPLP doses as low as 2 μg pDNA (~30 μg total lipid), correlating with the development of anti-PEG Ab response in SPLP-treated animals. As such, we still observed hypersensitivity at dosing intervals greater than 28 days in animals with established anti-PEG IgG responses (Fig. 2). In contrast, priming mice with empty liposomes did not induce anti-PEG Ab (Fig. 2B) or hypersensitivity to subsequent SPLP treatment, although empty liposomes could trigger the anaphylactic reaction in animals presensitized with SPLP. Features of the anaphylactic response to PEGylated liposomes are summarized in Table 1.

In the mouse, one mechanism of anaphylaxis involves PAF [42]. This reaction can be initiated by the formation of IgG containing immune complexes in the blood that trigger excess PAF release from Fcγ receptor-expressing cells [42]. To test if this mechanism was responsible for the anaphylactic reaction to PEGylated liposomes, we treated SPLP-sensitized mice with PAF receptor antagonists immediately prior to a second administration of SPLP. Prophylactic treatment with PAF antagonist CV6209 or CV3988 inhibited the anaphylactic reaction to PEGylated liposomes at challenge doses that otherwise proved fatal in control mice. This suggests that the acute toxicities following repeat administration of PEGylated liposomes are due to the systemic release of PAF, triggered by immune complex formation involving anti-PEG Ab. Hypersensitivity in this model was not associated with excessive systemic cytokine release or significant elevations in plasma histamine or complement activation products (not shown).

Although PEGylated liposomes have not been reported to be immunogenic in humans, their iv administration is associated with hypersensitive infusion reactions in a substantial number of patients [43,44]. Unlike the reactions described here, these clinical events occur upon first exposure to the liposome and have been correlated with the activation of complement [45,46] and a rapid redistribution of the liposomes from the blood to the liver and spleen [43]. Naturally occurring Abs against lipid components have been implicated in this hypersensitive response [46] and it cannot be discounted that this may include preexisting anti-PEG Ab in some patients. In this regard, preliminary analyses of sera from healthy volunteers have identified low levels of anti-PEG reactivity in certain donors (A.J., unpublished data), a finding consistent with previous reports of naturally occurring anti-PEG IgM in a proportion of human subjects [47,48]. It appears therefore that the human B cell repertoire can generate anti-PEG Ab and suggests that the immunogenicity of PEGylated liposomes may become a clinically relevant

TABLE 1: Characteristics of the hypersensitive response following repeat challenge with SPLP containing PEG-S-DSG

First dose (Day 0)	Second dose (Day 7)	Reaction
SPLP 100 μg	SPLP 100 μg	Moderate–severe
SPLP 20 μg	SPLP 100 μg	Moderate–severe
SPLP 2 μg	SPLP 100 μg	Moderate–severe
SPLP 100 μg	SPLP 20 μg	Mild
Empty liposomes	SPLP 100 μg	No reaction
SPLP 100 μg	Empty liposomes	Moderate–severe
SPLP 100 μg	SPLP + PAF antagonists	No reaction

Mice were treated on day 0 (first dose) and day 7 (second dose) with DSG-SPLP or empty liposomes at 100, 20, or 2 μg pDNA or the equivalent lipid dose. Hypersensitive reactions were scored according to the severity of symptoms 10–60 min after the second dose as described under Materials and Methods.

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