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Premature Drug Release from Polyethylene Glycol (PEG)-Coated Liposomal Doxorubicin *via* Formation of the Membrane Attack Complex

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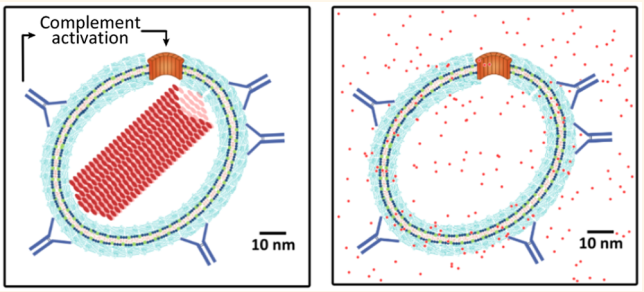
ABSTRACT: **Anti-polyethylene glycol (PEG) antibodies are present in many healthy individuals as well as in patients receiving polyethylene glycol-functionalized drugs. Antibodies against PEG-coated nanocarriers can accelerate their clearance, but their impact on nanodrug properties including nanocarrier integrity is unclear. Here, we show that anti-PEG IgG and IgM antibodies bind to PEG molecules on the surface of PEG-coated liposomal doxorubicin (Doxil, Doxisome, LC-101, and Lipo-Dox), resulting in complement activation, formation of the membrane attack complex (C5b-9) in the liposomal membrane, and rapid release of encapsulated doxorubicin from the liposomes. Drug release depended on both classical and alternative pathways of complement activation. Doxorubicin release of up to 40% was also observed in rats treated with anti-PEG IgG and PEG-coated liposomal doxorubicin. Our results demonstrate that anti-PEG antibodies can disrupt the membrane integrity of PEG-coated liposomal doxorubicin through activation of complement, which may alter therapeutic efficacy and safety in patients with high levels of pre-existing antibodies against PEG.**



KEYWORDS: *anti-PEG antibody, PEG-coated liposomal doxorubicin, PEGylated liposomal doxorubicin, complement, membrane attack complex, drug release*

Encapsulation of drugs can decrease side effects to normal tissues and improve clinical outcome.[1,2] However, nanomedicines can also produce toxicities such as infusion reactions and palmar−plantar erythrodysesthesia in patients treated with PEGylated liposomal doxorubicin.[3] Understanding toxicities associated with nanodrugs is important for safe and effective development of future nanomedicines.

Polyethylene glycol (PEG) is a biocompatible polymer often grafted to the surface of nanocarriers to decrease interactions with blood components, thereby increasing circulation times *in vivo*. Examples of clinically used PEGylated nanomedicines include Genexol-PM (polymeric micelle paclitaxel), Onivyde (PEGylated liposomal irinotecan), and Doxil (PEGylated liposomal doxorubicin). Several PEGylated nanomedicines are also under late-stage clinical development, including Lipoplatin (PEGylated liposomal cisplatin) and NK105 (paclitaxel in a polymer micelle).[4]

Although PEG is a widely used biocompatible polymer, antibodies that bind PEG are induced to high levels in some patients receiving PEGylated drugs that contain nucleic acids or nonhuman proteins.[5−8] Anti-PEG antibodies can also be induced in animal models after repeated injections of PEGylated compounds including PEGylated liposomes.[9−13] Pre-existing anti-PEG antibodies are also found in individuals that have never received PEGylated drugs. For example, a study conducted by our lab detected anti-PEG antibodies in 44.3% of a population of 1504 healthy Han Chinese.[14] Others have also found anti-PEG antibodies in healthy individuals

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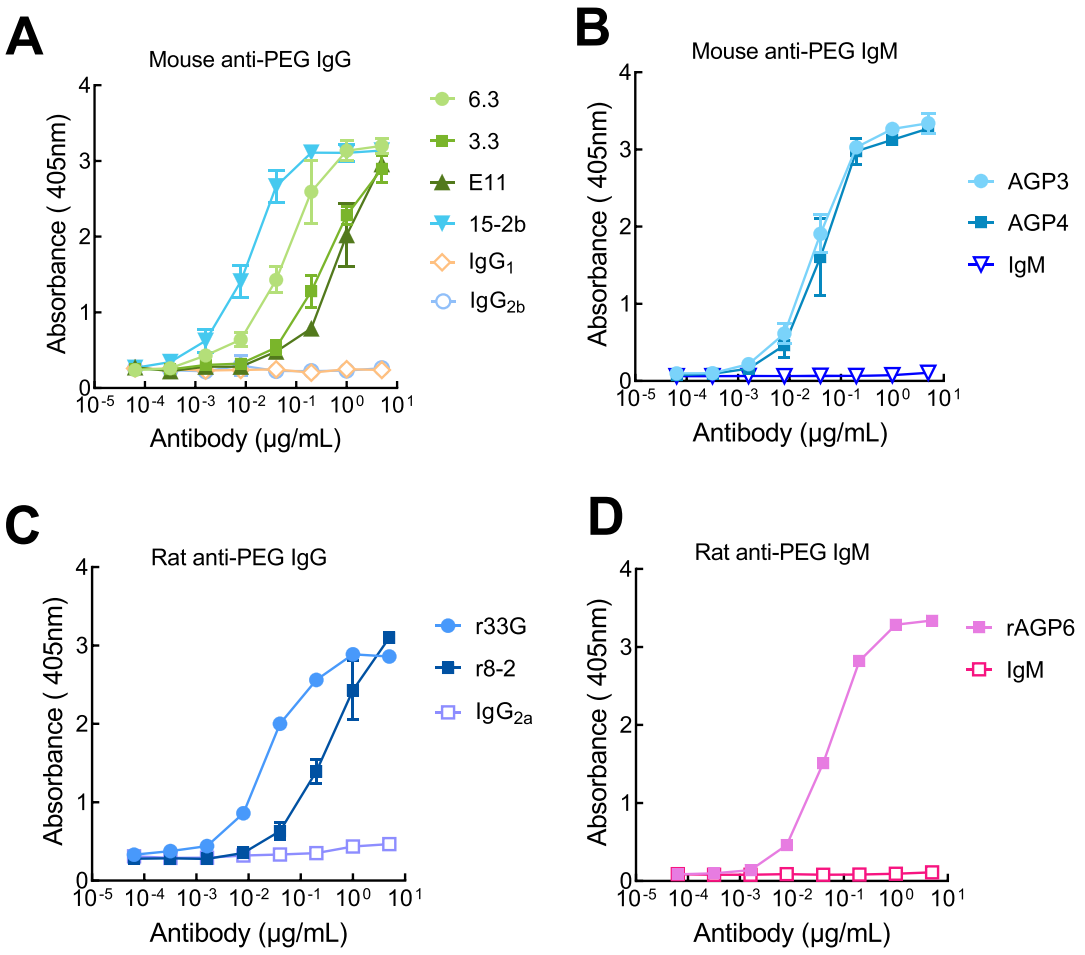

Figure 1. Binding of mouse and rat anti-PEG antibodies to Doxisome. Anti-PEG antibodies binding to immobilized Doxisome was determined by ELISA. (A) Mouse anti-PEG IgG antibodies 6.3, 3.3, and E11 and anti-mPEG antibody 15-2b. (B) Mouse anti-PEG IgM antibodies AGP3 and AGP4. (C) Rat anti-PEG IgG antibodies r33G and r8-2. (D) Rat anti-PEG IgM antibody rAGP6. Results show mean values of triplicate determinations. Isotype-matched control antibodies are also shown. Bars show standard deviations (SD).

across different age, sex, and ethnicity groups.[15] The presence of anti-PEG antibodies is associated with decreased therapeutic efficacy[5,8] and, in some cases, adverse events in patients.[6,7] However, little is currently known about possible effects of anti-PEG antibodies on the stability and structural integrity of PEGylated nanomedicines.

Antigen-bound antibodies can activate the classical pathway of complement to promote inflammatory responses against pathogens.[16] The end product of complement activation is the formation of the membrane attack complex (hereafter referred to as C5b-9), a barrel-like protein complex that can insert into lipid membranes of pathogens. C5b-9 forms a small channel that causes efflux of cellular contents into the environment, ultimately resulting in cellular death.[17] We therefore hypothesized that anti-PEG antibodies may bind to PEGylated liposomes to reduce the integrity of PEGylated liposomal doxorubicin (PLD) *via* activation of the complement cascade. This may accelerate the release of encapsulated drug molecules.

C5b-9 has been demonstrated to insert into nonliving membranes as well as cellular targets,[18−22] and studies have suggested that complement can be activated against PEGylated liposomes.[10,23−25] In addition, repeated low-dose injections of liposomes loaded with epirubicin were reported to generate anti-PEG IgM antibodies that accelerated clearance and drug release from liposomes.[26] However, it remains unknown if C5b-9 can insert into a lipid bilayer that is coated with a layer of PEGylated lipids. PLD typically contains 5 mol % PEG-modified lipids, in which the PEG chains form a brush configuration on the liposome surface that is believed to stabilize liposomes by combined dehydration of the lipid headgroup region along with increased hydration of the outer PEG layer.[27] Serum proteins can interact with and adsorb to PEGylated liposomes, but whether a protein pore can form on the surface of a PEGylated liposome is unknown.[28,29] In addition, it is unknown if an adequate density of anti-PEG IgG antibodies can bind in the proper orientation to the flexible PEG chains on PEGylated liposomes to initiate activation of the classical complement pathway, since multiple IgG molecules in close proximity are required for effective binding to C1q, the first step of the classical complement cascade.[30]

Using recently developed and previously characterized anti-PEG antibodies produced in our lab, we show that release of doxorubicin from Doxisome, Lipodox, and Doxil occurs in the presence of anti-PEG antibodies in both rat and human serum. Our data suggests that patients who have high levels of pre-existing anti-PEG antibodies could experience altered pharmacokinetics and therapeutic efficacy when administered PLD compared to patients without circulating anti-PEG antibodies.

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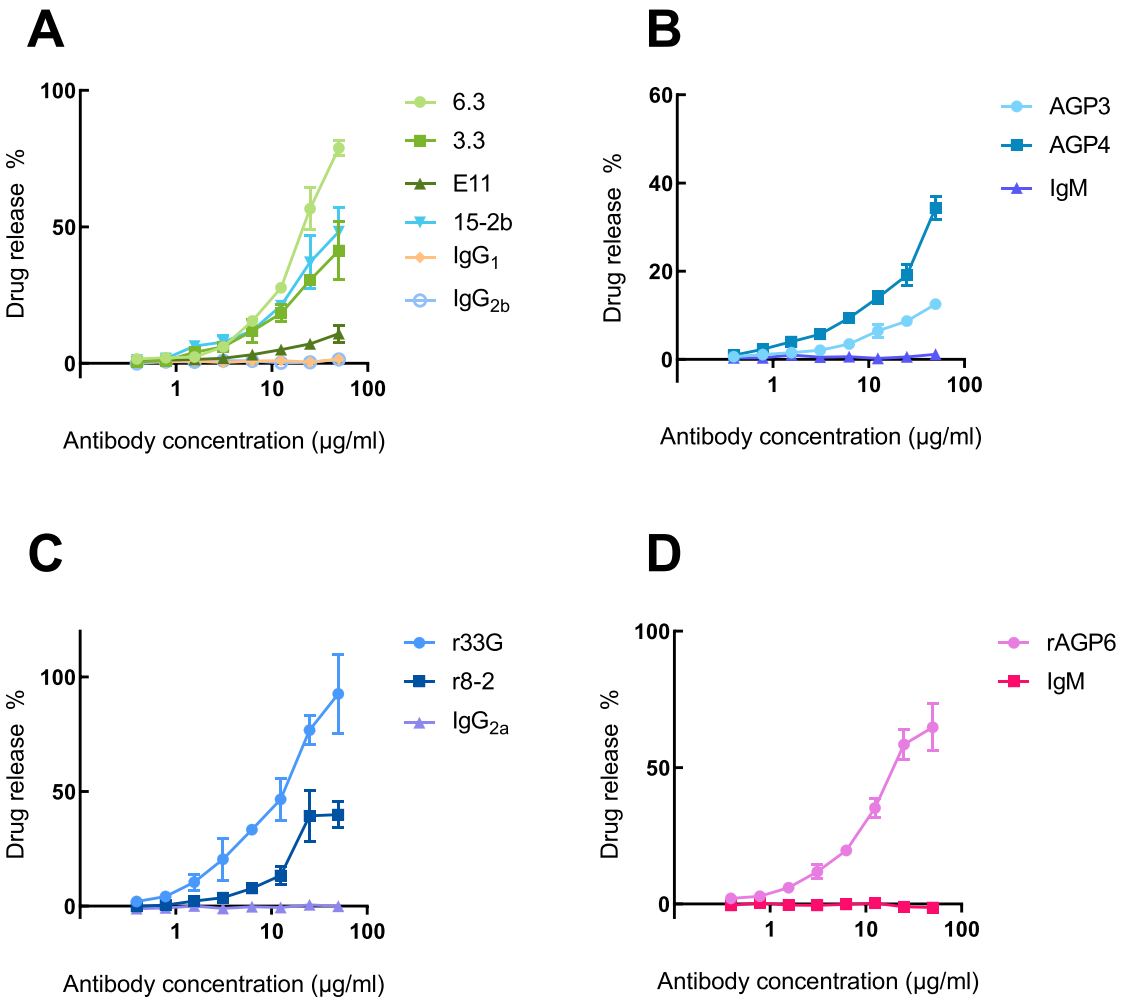

Figure 2. Monoclonal anti-PEG antibodies can induce drug release from PLD in rat serum. Monoclonal anti-PEG antibodies were serially diluted and incubated with rat serum and 10 μg/mL of Doxisome for 30 min before fluorescence of released doxorubicin was measured. (A) Monoclonal mouse anti-PEG IgG antibodies. (B) Monoclonal mouse anti-PEG IgM antibodies. (C) Monoclonal rat anti-PEG IgG_{2a} antibodies. (D) Monoclonal rat anti-PEG IgM antibodies. Results show mean percentage of doxorubicin released from Doxisome at each antibody concentration.

RESULTS AND DISCUSSION

Binding of Mouse and Rat Anti-PEG Antibodies to PEGylated Liposomal Doxorubicin. We previously generated a panel of mouse IgG and IgM monoclonal antibodies that can bind to PEG (Supplemental Table 1).[31−33] We first characterized binding of the antibodies to PLD by immobilizing Doxisome in ELISA plates coated with Hu6.3 (a human anti-PEG antibody)[34] before serial dilutions of the anti-PEG antibodies were added. Binding of the anti-PEG antibodies to Doxisome was determined by adding appropriate horseradish peroxidase conjugated secondary antibodies. All mouse anti-PEG IgG (Figure 1A) and IgM (Figure 1B) antibodies bound to immobilized Doxisome. 15-2b, which binds to the methoxy ends of PEG, bound most avidly to Doxisome with a half-maximal binding (EC_{50}) value of 0.07 nM (Supplemental Table 2). The other anti-PEG monoclonal antibodies, which bind to the repeating ethylene oxide subunits of the PEG backbone, bound to Doxisome with EC_{50} values ranging from 0.41 to 5 nM. Mouse anti-PEG IgM antibodies AGP3 and AGP4 bound to Doxisome with EC_{50} values of about 0.04 nM, reflecting the high avidity of IgM for PEGylated liposomes. We recently generated rat monoclonal antibodies that bind to the PEG backbone. Rat IgG antibody r33G bound to Doxisome with high apparent avidity (EC_{50} = 0.16 nM), whereas rat IgG r-82 bound less strongly (EC_{50} = 2.6 nM) (Figure 1C). Rat IgM antibody rAGP6 bound to Doxisome with similar avidity to mouse IgM antibodies (EC_{50} = 0.05 nM) (Figure 1D). Taken together, these results demonstrate that anti-PEG antibodies with different affinities and binding specificities can bind to PLD.

Mouse and Rat Anti-PEG Antibodies Can Induce Doxorubicin Release from Doxisome. We examined if anti-PEG antibodies can affect the stability of PLD by measuring doxorubicin fluorescence. Doxorubicin displays low fluorescence inside the lumen of liposomes due to self-quenching but substantially greater fluorescence when released from liposomes.[35] Incubation of Doxisome in mouse serum in the presence of mouse anti-PEG antibodies did not appear to destabilize Doxisome in comparison to nonbinding control antibodies (Supplemental Figure 1). However, many common mouse strains display low complement activity.[36] We therefore examined Doxisome stability by incubating serial dilutions of anti-PEG antibodies with a fixed concentration of Doxisome diluted in rat serum. We detected release of doxorubicin as measured by increased fluorescence when Doxisome was

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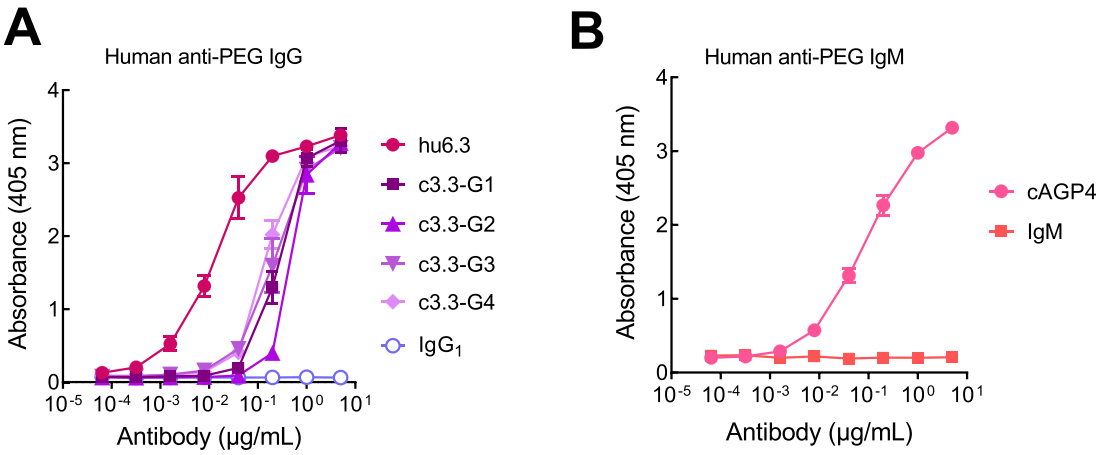

Figure 3. Binding of humanized anti-PEG antibodies to PLD. Humanized anti-PEG antibody binding to immobilized Doxisome was determined by ELISA. (A) Humanized anti-PEG IgG. (B) Chimeric anti-PEG IgM. Results show mean values of triplicate determinations. Binding of isotype-matched control antibodies is also shown. Bars show standard deviations (SD).

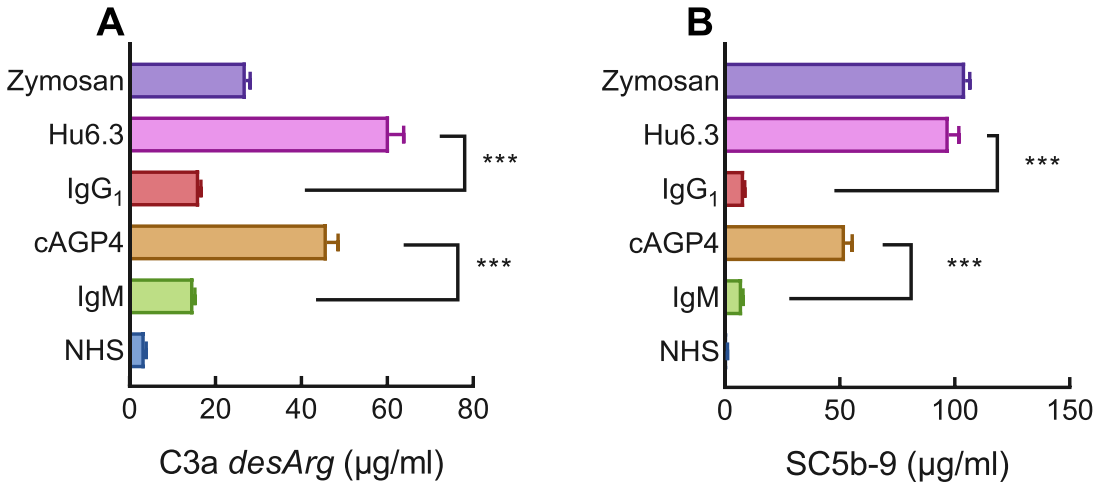

Figure 4. Humanized anti-PEG antibodies can activate complement in the presence of PLD. Humanized anti-PEG antibodies (Hu6.3 and cAGP4) and their respective isotype controls (IgG_1 and IgM) were incubated at 50 μg/mL with 10 μg/mL of Doxisome and 50% human serum for 30 min. Zymosan-treated human serum was used as a positive control for complement activation, while untreated human serum (NHS) was used as a negative control. Samples were diluted appropriately, and the concentrations of (A) human C3a and (B) human SC5b-9 were measured with commercial ELISA kits. Results show mean values ± SD. Significance is reported as $p < 0.001$ (***).

incubated with mouse anti-PEG IgG (Figure 2A) or mouse anti-PEG IgM (Figure 2B). Release of doxorubicin depended on the concentration of anti-PEG antibodies with greater release observed at higher antibody concentrations. Rat anti-PEG IgG (Figure 2C) and rat anti-PEG IgM (Figure 2D) antibodies also induced the release of doxorubicin in a dose-dependent manner. Doxisome destabilization required antibody binding to PEG, because isotype-matched nonbinding control antibodies did not induce significant release of doxorubicin from liposomes.

Release of doxorubicin was similar for the highest avidity antibodies (6.3, 15-2b, and r33G) but was substantially reduced for the lower avidity antibodies E11 and r8-2. Indeed, we found a significant correlation between anti-PEG IgG binding avidity and liposome drug release (Supplemental Figure 2). Treatment of serum with cobra venom factor (CVF) to deplete complement[37] or heat treatment to inactivate complement[38] both significantly reduced release of doxorubicin from Doxisome in the presence of anti-PEG antibodies, suggesting that destabilization of liposomes by anti-PEG antibodies was complement dependent (Supplemental Figure 3).

Humanized Anti-PEG Antibodies Also Bind PLD. To examine whether the phenomenon seen in rat serum translated to humans, humanized anti-PEG antibodies were generated (Supplemental Table 1). Hu6.3 is a totally humanized IgG_1 antibody that binds to the PEG backbone. c3.3-G1, c3.3-G2, c3.3-G3, and c3.3-G4 are chimeric antibodies based on murine 3.3 with human IgG_1, IgG_2, IgG_3, and IgG_4 constant regions, respectively. Hu6.3 displayed greater binding avidity for Doxisome in comparison to chimeric 3.3 antibodies (Figure 3A). c3.3-G_2 bound slightly less strongly to Doxisome as compared to c3.3 antibodies with other heavy chain constant regions. A chimeric IgM antibody (cAGP4) with a human IgM heavy chain constant region also bound well to Doxisome (Figure 3B). These results show that humanized and chimeric human anti-PEG antibodies can also bind to Doxisome.

Human Anti-PEG Antibodies Bound to PLD Can Activate Complement. To verify that anti-PEG antibodies bound to PLD activate complement, we assayed C3a *desArg*

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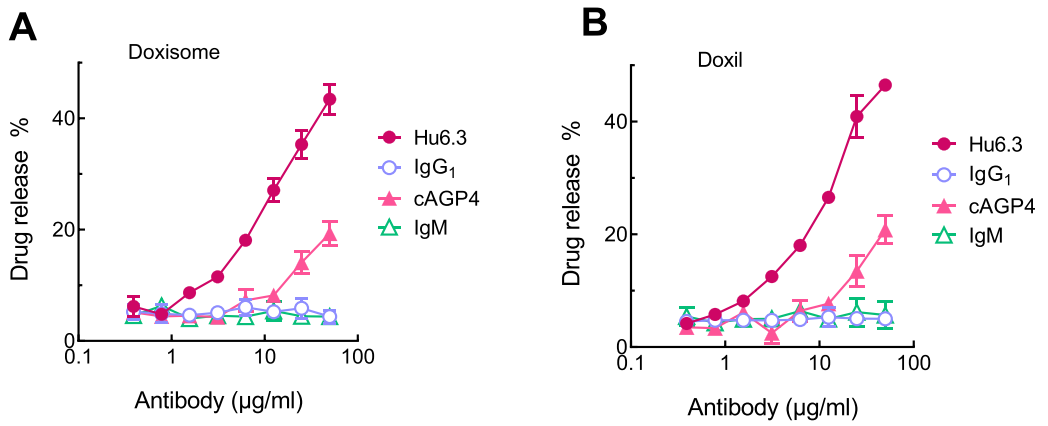

Figure 5. Humanized anti-PEG antibodies can induce similar doxorubicin release from Doxisome and Doxil in human serum. Humanized anti-PEG antibodies were serially diluted in GHBS^{+2} and human serum containing 10 μg/mL of (A) Doxisome or (B) Doxil. Mixtures were incubated for 30 min before fluorescence of released doxorubicin was measured.

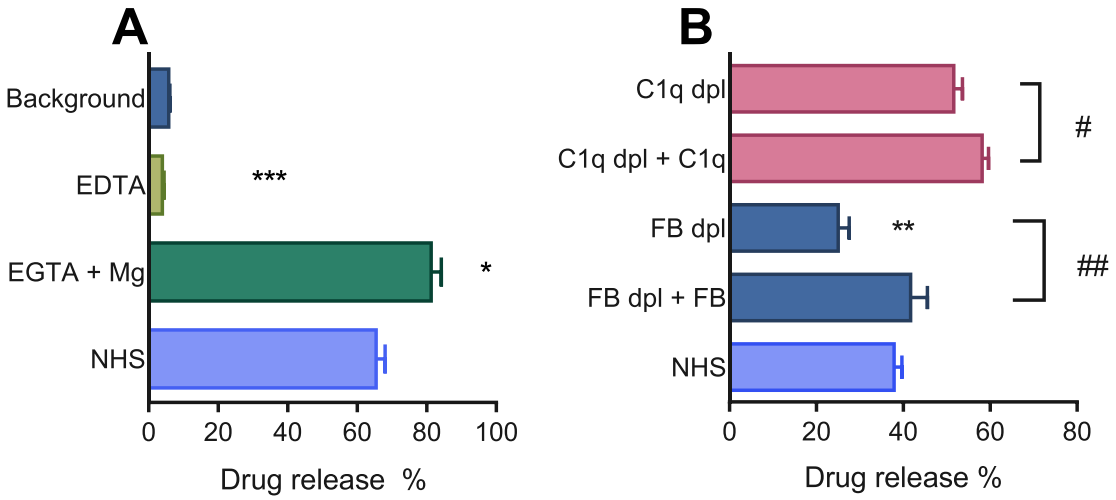

Figure 6. Humanized anti-PEG IgG on PLD activates complement primarily through the alternative pathway. Hu6.3 (50 μg/mL) was incubated with 10 μg/mL Doxisome in the presence of (A) human sera treated with chelating agent EDTA or EGTA+Mg or (B) human sera depleted of complement proteins. Respective depleted proteins were added back into the reaction after 15 min of incubation. Bars show SD. Significant difference between NHS and depleted serum is indicated as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Significant difference between indicated groups is indicated as $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$.

and SC5b-9, two important markers of complement activation that are common to all three complement pathways (classical, lectin, and alternative). C3a, an anaphylatoxin, is formed in the earlier steps of complement activation, when C3 convertase cleaves C3 into the larger fragment C3b and smaller fragment C3a; C3a is quickly hydrolyzed into the more stable form, C3a *desArg*. SC5b-9 is the soluble nonfunctional form of C5b-9 and is directly proportional to C5b-9 concentrations.

Human anti-PEG IgG (Hu6.3) and IgM (cAGP4) antibodies generated significantly higher levels of C3a in the presence of human serum and Doxisome as compared to control nonbinding human IgG and IgM antibodies (Figure 4A). Similar results were observed for SC5b-9, which increased significantly in the presence of Doxisome and anti-PEG antibodies compared to non-PEG-binding antibodies (Figure 4B). These results show that both anti-PEG IgG and IgM antibodies, when bound to PEG on the surface of PLD, can activate complement.

Human Anti-PEG Antibodies Can Cause Drug Release from PLD. We tested if human anti-PEG antibodies can also destabilize PLD. Humanized anti-PEG antibodies induced similar release of doxorubicin from Doxisome (Figure 5A) and Doxil (Figure 5B) in the presence of human serum. Hu6.3 at 50 μg/mL triggered around 40% of doxorubicin to be released from both Doxisome and Doxil (Supplemental Figure 4). Human anti-PEG IgG and IgM antibodies also produced similar destabilization of Lipo-Dox, a PLD that uses distearoylphosphatidylcholine (DSPC) rather than HSPC found in Doxisome and Doxil (Supplemental Figure 5). A human serum sample from a donor positive for pre-existing anti-PEG antibodies (7.5 μg/mL) also causes doxorubicin release from PLD (Supplemental Figure 6). When human serum was pretreated with CVF or heat-inactivated, the effect of the added antibodies was attenuated (Supplemental Figure 3C). These results indicate that the release of doxorubicin from PLD is reliant on both anti-PEG antibodies and complement proteins in serum and that drug release is unaffected by the different liposomal compositions of the examined PLD.

The Alternative Pathway Is Important for PLD Destabilization by Anti-PEG Antibodies. Complement can be activated by three pathways: the classical pathway, which is activated by the binding of antibodies to the surface of

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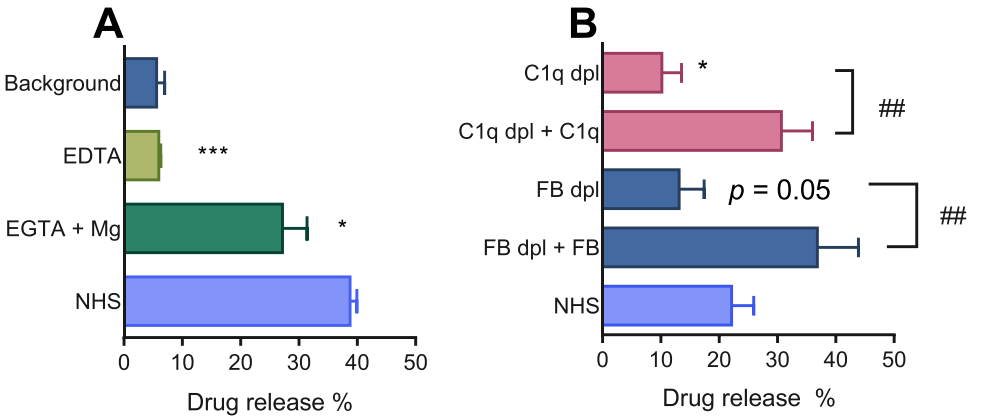

Figure 7. Humanized anti-PEG IgM on PLD activates complement through both the classical and alternative pathways. cAGP4 (50 μg/mL) was incubated with 10 μg/mL Doxisome in the presence of (A) human sera treated with chelating agent EDTA or EGTA+Mg or (B) human sera depleted of complement proteins. Respective depleted proteins were added back into the reaction after 15 min of incubation. Bars show SD. Significant differences between NHS and depleted serum is indicated as *, $p < 0.05$; ***, $p < 0.001$. Significant differences between indicated groups is indicated as ##, $p < 0.01$.

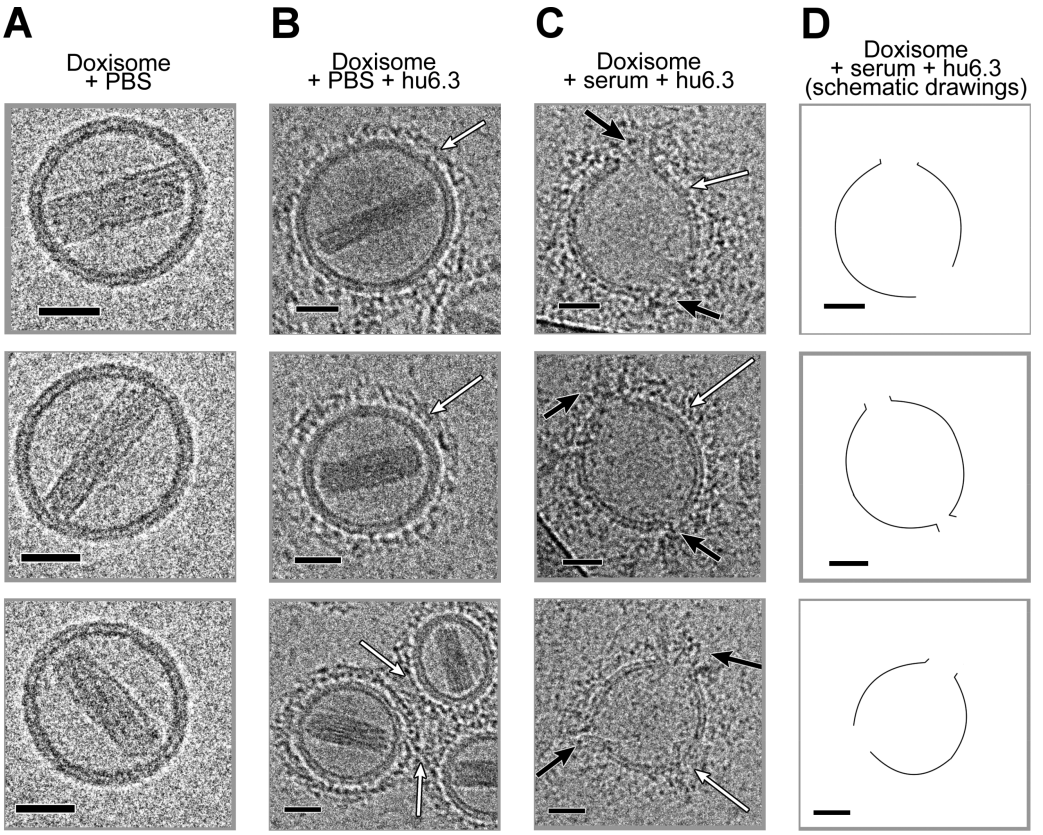

Figure 8. Cryo-EM images of Doxisome. (A) Doxisome in PBS. (B) Doxisome in PBS with 50 μg/mL Hu6.3. (C) Doxisome in human serum and 50 μg/mL Hu6.3. (D) Schematic drawings of the C5b-9 complex embedded into the liposomal membranes seen in (C). Black arrows point to C5b-9 channels on the surface of Doxisome, and white arrows point to the anti-PEG antibody corona. Scale bars represent 25 nm.

pathogens; the lectin pathway, which is initiated by the binding of complement proteins to carbohydrates on pathogens; and the alternative pathway, which represents a default and continuous source of complement activation in the absence of complement inhibitors.[39,40] The classical and lectin pathways are calcium dependent, whereas the alternative pathway requires magnesium ions. To investigate the major mechanism of complement activation by anti-PEG antibodies on PDL, we used EDTA to deplete divalent ions and block all pathways of complement activation or EGTA supplemented with Mg^{2+} to deplete Ca^{2+} ions and selectively block the classical and lectin pathways.[41] EDTA significantly reduced drug release induced by Hu6.3 anti-PEG IgG to near background levels (Figure 6A), consistent with complement-mediated drug release. By contrast, drug release was restored in serum treated with EGTA+Mg, suggesting that complement activation by anti-PEG IgG on PLD occurred *via* the alternative pathway.

The mechanism of complement activation by human anti-PEG IgG was further examined by measuring drug release in

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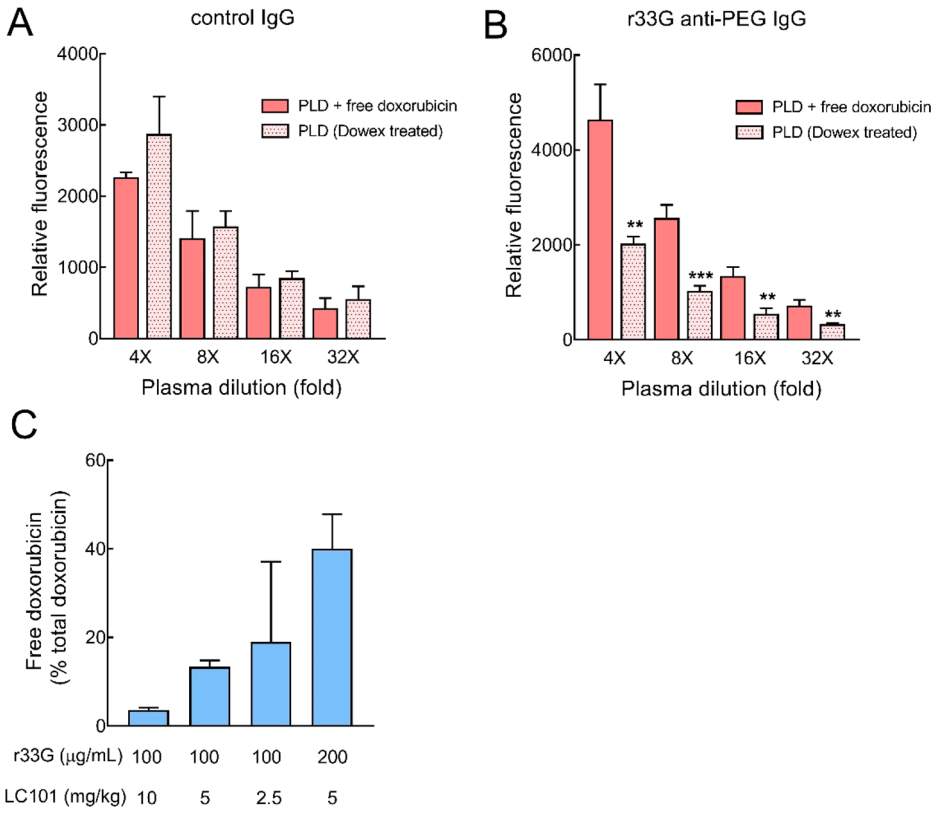

Figure 9. Anti-PEG IgG can induce accelerated release of doxorubicin from PLD in rats. Groups of three Wistar rats were intravenously injected with control rat IgG (A) or r33G rat anti-PEG IgG (B) before intravenous administration of 5 mg/kg PLD. The fluorescence of serially diluted plasma samples taken within 5 min of PLD administration before (solid columns) or after (dotted columns) removal of free doxorubicin on Dowex cationic ion-exchange resin is shown. Significant difference in mean fluorescence values of plasma before or after removal of free doxorubicin is indicated: **, $p < 0.01$; ***, $p < 0.001$. (C) Amounts of free doxorubicin in plasma of rats as a percentage of total doxorubicin (free plus encapsulated) in plasma after sequential intravenous injection of r33G anti-PEG IgG to give the indicated serum concentrations and 5 or 10 mg/kg LC-101 PEGylated liposomal doxorubicin. The results show mean values from serial plasma dilutions isolated from 2, 3, 3, and 1 rat, respectively.

human sera depleted of C1q (classical pathway) or factor B (alternative pathway). Depletion of C1q did not reduce lysis as compared to normal human serum, and reconstitution of C1q only slightly elevated drug release, indicating a minor role at most for the classical pathway of complement activation in liposome destabilization by Hu6.3 anti-PEG IgG. By contrast, depletion of factor B significantly reduced drug release (Figure 6B). When factor B was reconstituted to physiological concentrations, drug release significantly increased. Thus, PLD destabilization by anti-PEG IgG appears to be more dependent on the alternative pathway than the classical pathway of complement activation.

In contrast to Hu6.3, both EDTA and EGTA+Mg significantly reduced drug release from Doxisome caused by cAGP4, suggesting a role for the classical pathway in destabilizing PLD in the presence of anti-PEG IgM (Figure 7A). Drug release from PLD induced by cAGP4 was reduced in serum that was depleted of either C1q or factor B (Figure 7B). Reconstitution of depleted serum with C1q or factor B significantly increased release of doxorubicin from PLD in the presence of cAGP4 (Figure 7B). Together, these data are consistent with cAGP4 activating complement on PLD by both classical and alternative pathways.

Cryo-EM Imaging. We employed cryo-EM to visually verify release of doxorubicin from the lumen of liposomes and examine if C5b-9 pores could be observed in the liposomal lipid bilayer. Cryo-EM photos of Doxisome in PBS without addition of antibodies or serum show that more than 99% of the liposomes have an intact lipid membrane and drug nanorod crystals (Figure 8A). When Hu6.3 anti-PEG IgG was added in PBS, clear clusters of bound antibodies were visible around the liposomes (indicated by white arrows, Figure 8B). In the absence of serum, however, most of the liposomes remained intact with drug crystals inside the liposome lumen. By contrast, when Hu6.3 was added to Doxisome in the presence of human serum, liposomes displayed loss of doxorubicin nanorods as well as a dense layer of bound antibodies (Figure 8C). Clear channels were evident on some liposomes, corresponding to C5b-9 complexes present in the lipid bilayer (Figure 8C, black arrows). Schematic drawings of the discontinuous membrane and channels in the liposomes are shown in Figure 8D.

Heat inactivation of complement activity in serum prevented the formation of C5b-9 channels and drug release even though Hu6.3 anti-PEG antibodies were clearly present on the surface of the liposomes (Supplemental Figure 7A). Replacement of Hu6.3 with a negative-control human IgG_1 dramatically reduced the loss of doxorubicin from liposomes, consistent with the need for specific antibody binding to the liposome surface for efficient generation of C5b-9 complexes (Supplemental Figure 7B).

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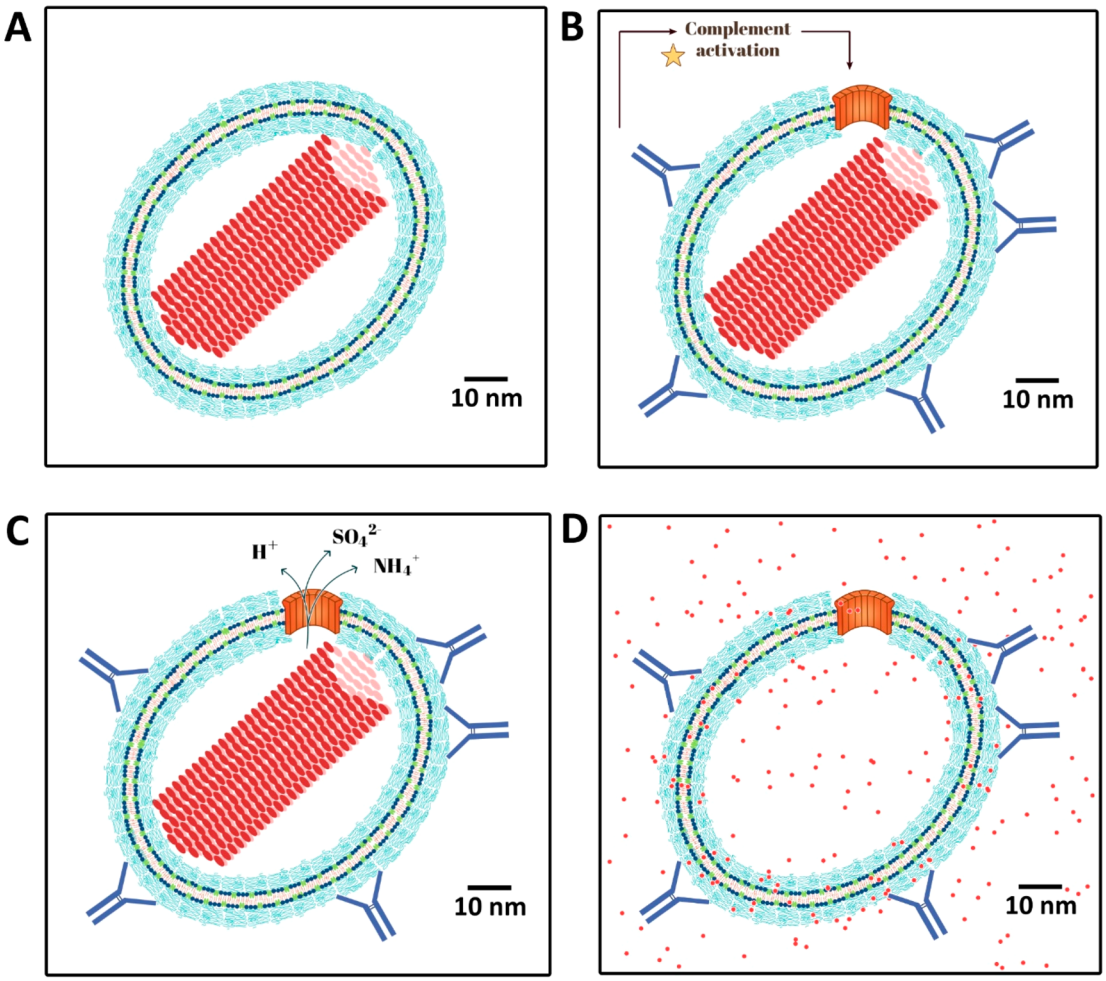

Figure 10. Proposed mechanism of PLD destabilization by anti-PEG antibodies. (A) PEGylated liposomal doxorubicin under normal conditions. (B) Anti-PEG antibodies that bind to PEG on the surface of PLD activate complement and form a C5b-9 channel in the liposome membrane (orange). (C) Ammonium, sulfate, and protons rapidly leak out of the pore formed by C5b-9. (D) Collapse of the proton and ammonium gradients leads to doxorubicin (red) dissolving back into aqueous solution and diffusing out of the PEGylated liposomal carrier into the surrounding environment.

The proportion of empty liposomes in comparison to the number of liposomes in which the doxorubicin nanorod was present was determined by visually inspecting wide-view cryo-EM images (Supplemental Figure 8). In agreement with the fluorescence release data, doxorubicin nanorod crystals were absent in almost 80% of liposomes incubated with human 6.3 anti-PEG IgG in the presence of human serum (Figure 8C; Supplemental Figure 9). Empty liposomes were rarely observed if complement was not present (Figure 8B) or if complement was heat inactivated (Supplemental Figure 7A). A small fraction of empty liposomes was observed when an equal concentration of control human IgG was incubated with Doxisome in the presence of serum, suggesting background lysis of liposomes by complement activated by the alternative pathway.

Overall, the cryo-EM images support our fluorescence-detected drug release data: that drug leakage depended on both anti-PEG antibodies and intact complement proteins. Additionally, wide-view images (Supplemental Figure 8) also revealed that liposomes tended to aggregate when Hu6.3 antibody was present, suggesting that individual Hu6.3 antibodies can simultaneously bind to different liposomes. This suggests another mechanism by which anti-PEG antibodies might impact the distribution of PEGylated nanoparticles *in vivo*. In conclusion, formation of C5b-9 in the lipid bilayer of PLD is dependent on both anti-PEG antibodies and complement proteins in serum.

***In Vivo* Drug Release.** We examined if anti-PEG antibodies could induce drug release from PLD in a rat model since complement activation and doxorubicin leakage were observed in the presence of anti-PEG antibodies in rat serum *in vitro*. Rats were first i.v. injected with either r33G rat anti-PEG IgG or a negative control rat IgG to mimic the presence of pre-existing anti-PEG antibodies in the circulation. After 30 min, the rats were i.v. injected with 2.5, 5, or 10 mg/kg LC-101, a PEGylated liposomal formulation with a similar composition to Doxil. Since *in vitro* studies demonstrated that anti-PEG antibodies could induce very rapid release of doxorubicin from PLD (Supplemental Figure 11) and doxorubicin displays rapid elimination kinetics,[42] we collected blood samples from rats into K2-EDTA tubes within 5 min of PLD administration to immediately quench further complement activation. Serially diluted plasma was then either untreated (and therefore contains both PLD and released doxorubicin) or treated with Dowex strong cationic ion-exchange resin to adsorb free doxorubicin (and therefore

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contain only intact PLD). Serially diluted plasma was examined to ensure that Dowex adsorption of doxorubicin was not saturated. Plasma from rats treated with control IgG and LC-101 displayed higher fluorescence after treatment with Dowex (Figure 9A), consistent with absorption of water from plasma during rehydration of the added Dowex resin, resulting in an apparent increase in PLD concentration due to reduced plasma volume. By contrast, plasma from rats treated with r33G anti-PEG IgG and LC-101 displayed significantly lower fluorescence after treatment with Dowex (Figure 9B), indicating the removal of free doxorubicin from the plasma samples by Dowex. Estimation of the amount of free doxorubicin as a percentage of total doxorubicin (free plus encapsulated in liposomes) in plasma samples under the experimental conditions examined (Supplemental Figure 14A–D) gave values ranging from a few percent to about 40% (Figure 9C). These results demonstrate that anti-PEG IgG can induce rapid release of doxorubicin from PLD in rats. Of interest, rats that received r33G and PLD but not control IgG and PLD experienced signs of distress including lack of movement and a dazed appearance.

In our study, incubating anti-PEG antibodies with sera and PLD induced drug release. The ability of the membrane attack complex to target nonliving, noncellular membranes was established as early as the 1970s, when researchers showed ultrasound images of C5b-9 forming 11 nm lesions on the membranes of liposomes.[17−20] Others have also immobilized C5b-9s onto lipid bilayers or liposomes for structural imaging.[18,19,21,22] PEGylated liposomes, however, are decorated with 5 mol % PEG-lipid. It was therefore unclear whether C5b-9 can penetrate the PEG steric shield and insert into the liposomal membrane. We show through measuring fluorescence in drug release assays that PEG does not prevent formation of functional C5b-9 on PLD. Fluorescence increased in a dose-dependent manner when both anti-PEG IgG and IgM were incubated with sera and PLD, except for humanized anti-PEG IgG_4 ($c3.3\text{-}G_4$), which induced less drug release than the other subclasses of humanized anti-PEG IgG (Supplemental Figure 10). This is consistent with studies showing that human IgG_4 ineffectively activates complement.[43,44] Drug release was inhibited when anti-PEG antibodies were replaced with negative control antibodies or if the serum was pretreated to inactivate complement proteins (Supplemental Figure 7). This phenomenon was further confirmed through cryo-EM, where the doxorubicin crystals are no longer visible after incubation with human anti-PEG IgG and human serum; C5b-9 and open lesions are clearly observed in some liposomes. Taken together, our data show that PEGylation of liposomes cannot prevent C5b-9 formation on the membrane surface; furthermore, these C5b-9 complexes are functional and can accelerate drug release from liposomes *in vitro*.

The mechanism for the rapid release of doxorubicin is likely related to disruption of the proton and ammonium ion gradients in the liposomes. Doxorubicin is remotely loaded into liposomes by creation of a transmembrane ammonium sulfate gradient in which the internal ammonium sulfate concentration is approximately 250 mM.[45] The internal sulfate anions induce precipitation of doxorubicin as a sulfate salt, which can be visualized as a single nanorod crystal that is approximately 70 nm long and 20 nm wide.[46] Previous studies have demonstrated that disruption of the ammonium gradient with the ionophore nonactine or collapse of the pH gradient by the ionophore nigericin induces fast and total release of doxorubicin from Doxil.[45] The relatively large C5b-9 channel (~5−11 nm in diameter depending on the number of C9 molecules incorporated into the membrane complex)[47] will likewise disrupt the pH and ammonium gradients, thereby allowing rapid dissolution of doxorubicin sulfate crystals.[46] Solubilized doxorubicin can then rapidly diffuse through the lipid bilayer of the liposome (Figure 10). Drug release occurs very rapidly; fluorescence measurements at 15 s intervals showed that drug release occurs almost instantaneously (Supplemental Figure 11) and reached plateau fluorescence in less than 10 min. Furthermore, the fluorescence data indicate that Hu6.3 anti-PEG IgG can cause release of up to 30−40% of total drug from Doxisome (Figure 5A); however, the cryo-EM images suggest a binary event in which either liposomes have doxorubicin-sulfate crystals (usually one crystal inside each liposomes) or these crystals are absent (Figure 9; Supplemental Figure 8). These data further support an ammonium ion/proton gradient collapse followed by rapid diffusion of doxorubicin as the main mechanism for drug leakage.

An important question raised by our study is if there are sufficient concentrations of pre-existing anti-PEG antibodies in some individuals to form membrane attack complexes and cause premature drug release from PLD. Our *in vitro* studies using human anti-PEG antibodies found that 20% of internal doxorubicin was released from 10 μg/mL PLD in the presence of about 7 μg/mL anti-PEG IgG or 50 μg/mL anti-PEG IgM (Figure 6). This corresponds to approximately 56 molecules of IgG or 60 molecules of IgM per liposome. At a typical PLD serum concentrations of 8.3 μg/mL,[48] the estimated concentrations of anti-PEG IgG and IgM required to reach these levels are 5.8 μg/mL for anti-PEG IgG and 42 μg/mL for anti-PEG IgM. Based on our study of pre-existing anti-PEG antibodies in 1400 normal donors[14] (which we have subsequently confirmed with an additional 900 donor samples (unpublished data)), we estimate that about 5.5% of normal individuals have pre-existing anti-PEG IgG antibody levels that equal or exceed this cutoff, whereas less than 0.1% of individuals have anti-PEG IgM concentrations exceeding 42 μg/mL. On the other hand, preliminary studies in rats showed that about 20% of doxorubicin was released from PLD in the presence of 130 μg/mL rat anti-PEG IgG that was administered to the rats prior to PLD administration. An additional complication is that anti-PEG antibodies can also accelerate the clearance of PLD, at least in animal models.[49] The kinetics of complement activation and accompanying drug release and accelerated blood clearance of PLD occur on relatively similar time scales, suggesting that drug release and PLD clearance may interact in a complicated fashion. Most studies that have focused on the effect of anti-PEG IgM antibodies on PLD.[10,50,51] However, our results in the present study as well as recent studies showing that anti-PEG IgG can induce accelerated blood clearance of PLD suggest that more attention should be paid to anti-PEG IgG antibodies.[14,15,52]

Clinical trials measuring the pharmacokinetic properties of PLD have not reported accelerated clearance of doxorubicin since 1997 when the internal ammonium sulfate concentration was increased to enhance the stability of doxorubicin in the liposomes.[53] The Dowex 50 cation exchanger method was developed to differentiate between liposome-encapsulated and free doxorubicin.[54,55] This approach was used in the first clinical study of Doxil, where it showed lack of significant release of doxorubicin in patient's blood, as there was no

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difference in the amount of drug in plasma samples before and after treatment with Dowex 50 cation exchanger.[56] The reasons for the disparity between the results of our *in vitro* and *in vivo* studies and clinical data are unclear, but it is possible that the concentrations of anti-PEG antibodies that destabilize liposome integrity also induce infusion reactions in patients. Since drug administration is stopped or delayed in patients that exhibit infusion reactions, their data are excluded in pharmacokinetic analysis. Support for this idea comes from studies showing that a wide range of murine anti-PEG antibodies can activate complement in the presence of PLD and studies in pigs which correlate infusion reactions to nanoparticles with complement activation.[57−59] Indeed, it was recently reported that anti-PEG IgM can activate complement and induce infusion reactions in pigs after PLD administration.[60] In addition, patients are often pretreated to prevent possible infusion reactions, which might affect release of drug from liposomes. We also used a bolus injection of PLD in rats, whereas patients usually receive a slow infusion of PLD, which might alter the interactions between anti-PEG antibodies and PLD. Anti-PEG antibodies in patients are also polyclonal and might display different affinities and binding specificities as compared to the monoclonal antibodies used in our studies. The significance of complement-mediated destabilization of PLD in patients therefore requires further clinical investigation.

Plasma complement is activated in one of three ways: by the classical, alternative, or lectin pathways.[16] Antigen-bound antibodies are typically thought to activate the classical pathway of complement through their Fc regions. Binding to an antigen causes conformational changes in antibody structure that allow the CH2 regions of IgG and IgM to bind C1q, the hexameric molecule responsible for the first step of classical pathway activation.[61,62] However, results using ion chelators and complement-depleted serum revealed that anti-PEG IgG activated complement on PLD mainly through the alternative pathway. This finding is consistent with reports that the alternative complement pathway can be activated by PEG[24] and opsonized nanoparticles.[63] The inefficient activation of the classical complement pathway might be due to the requirement for physical binding of at least two (and optimally six) adjacent IgG molecules for effective C1q binding and activation.[30,64] The layer of PEG molecules on PLD may be too dynamic and flexible for effective C1q binding. Indeed, complement fixation is predicted to decrease when the fluidity of a membrane increases over a certain threshold.[65] By contrast, IgM-mediated drug lysis appeared to proceed *via* both the classical and alternative pathways. IgM may be able to activate the classical pathway on PLD because a single bound IgM pentamer can activate C1q. This is consistent with detectable doxorubicin release when about 1 μg/mL anti-PEG IgM was present, which corresponds to one molecule of IgM per liposome.

An appropriate animal model that mimics the human physiological system is crucial for complement studies. In the present research, we used male Wistar rat serum as an *in vitro* and *in vivo* model for complement-activated lysis of PLD for both mouse and rat monoclonal antibodies. Prior to using rat serum, we conducted preliminary drug release experiments in male BALB/c and C57 serum and observed no significant difference in the lysis ability of anti-PEG antibodies and their respective negative controls (Supplemental Figure 1). This result is consistent with previous reports of low cytolytic activity[66] or high levels of complement inhibitors[67] in mouse serum. Therefore, mice may not be a suitable model for studying complement-mediated liposome lysis. Based on Ong and Mattes' work describing the CH_{50} titer of serum in common laboratory animals,[36] we identified Wistar rats as potential candidates for an animal model of drug release assays, as their CH_{50} titers are similar to humans (7536 and 6666 units/mL, respectively). In our drug release assays, mouse anti-PEG antibodies were capable of lysing Doxisome in Wistar serum to a similar degree to human anti-PEG antibodies in human serum, suggesting that rats are a suitable model to study complement activation by anti-PEG antibodies. Although drug release was also detected in rats *in vivo*, interspecies differences will still have to be considered before the results can be translated to humans.

CONCLUSIONS

Here, we show that a wide range of anti-PEG antibodies can bind to PLD *in vitro*. Both anti-PEG IgG and IgM activated the complement cascade in the presence of PLD, resulting in the formation of C5b-9 in the liposomal membranes followed by rapid leakage of doxorubicin from PLD. Anti-PEG IgG also induced the rapid release of doxorubicin from PLD in rats. Allergy-like responses to PLD (CARPA) have been suggested to be related to complement activation.[25,68] These sometimes-serious side-effects may be related to the presence of anti-PEG antibodies in patients.[5−7,9,69,70] Our study suggests that further studies are warranted on the relationship between complement activation, drug release, and adverse events in patients receiving PEGylated liposomal doxorubicin.

MATERIALS AND METHODS

Materials. Doxil was a kind gift from Dr. Andre Lieber (University of Washington, Seattle, WA, USA). The lipid membrane of Doxil is composed of hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and *N*-(carbonylmethoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE) in a molar ratio of 56:38:5 encapsulating 2 mg/mL doxorubicin.[71] Doxisome, a kind gift from Taiwan Liposome Company Ltd., Taipei, is a generic form of Doxil and has the same composition as Doxil. LC-101 was kindly provided by Ayana Pharma and is also equivalent to Doxil. Lipo-Dox, purchased from TTY Biopharm Co., Ltd., also contains 2 mg/mL doxorubicin, but substitutes distearoylphosphatidylcholine (DSPC) in place of HSPC.[72] All vials of PLD were stored at 4 °C in foil-wrapped containers to prevent photobleaching.

NH_2-PEG_{10k}-NH_2 was from Iris Biotech GMBH (catalogue number: 24991-53-5). Detection antibodies for ELISAs and non-PEG-binding antibodies used in drug release assays are listed in Supplemental Table 3. MicroVue SC5b-9 Plus enzyme immunoassay (A020) and MicroVue C3a Plus EIA (A031) are from Quidel; assays were carried out according to the manufacturer's instructions. Complement protein-depleted sera (C1q-depleted serum, A509; factor B-depleted serum, A506) and purified complement proteins (C1q, A400; factor B, A408) were also purchased from Quidel.

Anti-PEG Antibodies. All anti-PEG antibodies used in this study are summarized in Supplemental Table 1. Anti-PEG antibodies 6.3, 3.3, E11, 15-2b, AGP3, AGP4, cAGP4, and c3.3-G_1 were generated in our lab as previously described.[14,32,73−75]

We created a fully humanized anti-PEG antibody (Hu6.3) based on the mouse anti-PEG antibody 6.3 by CDR grafting onto a human IgG_1 framework and constant region genes.[76] A series of human chimeric antibodies with different immunoglobulin G heavy chains were also generated by fusing human immunoglobulin G_2, G_3, or G_4 heavy chain genes to variable region genes of 3.3 (mouse anti-PEG IgG_1).

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Anti-PEG rat antibodies (r8-2, r33G, and rAGP6) were generated *via* a previously described method.[74] Briefly, SD rats were immunized with 150 μg of PEG_{5000} conjugated to *E. coli* beta glucuronidase (eβG-PEG_{5000}), then boosted every 4 weeks with 100, 75, 50, and 30 μg of eβG-PEG_{5000} in complete and incomplete Freund's adjuvant (Sigma-Aldrich; F5881 and F5506). Rat spleens were harvested, and cells were fused with BALB/c FO myeloma cells (ATCC; CRL-1646). Antibody isotype was determined by ELISA.

Animals. Male Wistar rats and C57BL/6 mice were purchased from Biolasco (Taipei, Taiwan). Male BALB/c mice were purchased from the National Laboratory Animal Centre (Taipei, Taiwan). Animals were housed under standard light/dark cycles and had access to food and water *ad libitum*. Animal experiments were conducted according to institutional guidelines and ethically approved by the Laboratory Animal Facility of the Institute of Biomedical Sciences, Academia Sinica.

Serum Collection. Tail artery blood was collected from male Wistar rats. Serum was processed to preserve complement activity.[77] Briefly, whole blood was clotted at room temperature for 30 min until clot retraction, then spun down at 3000*g* for 7 min to collect the serum. A second centrifugation was carried out at 16000*g* for 2.5 min to remove remaining white or red blood cells. Serum was collected and stored at −80 °C until use. To collect mouse serum, BALB/c and C57BL/6 mice were euthanized by isoflurane and blood was collected *via* cardiac puncture; serum was also processed as described for rat serum.

Human venous blood from eight healthy donors (2 females) was collected in uncoated venepuncture tubes without anticoagulants, and serum processed as described above to preserve complement activity.[78] All procedures were carried out according to approved protocols (IRB: AS-IRB02-108153).

Binding of Anti-PEG Antibodies to PLD. To compare the relative avidity of anti-PEG antibodies to PLD, Doxisome was captured in Corning 96-well EIA microplates (Sigma-Aldrich) coated with 1 μg/well human 6.3 antibody (to detect mouse and rat antibody binding) or mouse 6.3 antibody (to measure human antibody binding) overnight in coating buffer (0.05 M Na_2CO_3/0.05 M $NaHCO_3$; pH 8.5). After washing off excess antibody and blocking with 5% skim milk in PBS, 5 μg/well of Doxisome in 2% skim milk/PBS was incubated in the plates for 1 h. Plates were washed three times with PBS, and serial dilutions of anti-PEG antibodies were added to the plates for 1 h. Anti-PEG antibody binding to Doxisome was detected with HRP-conjugated secondary antibodies (Supplemental Table 3). Peroxidase activity was quantified by incubation of 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H_2O_2 (3000:1) and reading the optical density on a microplate reader at 405 nm. Non-PEG-binding isotype-matched antibodies were used as controls (Supplemental Table 3).

ELISA for Detecting Anti-PEG Antibodies in Human Serum. EIA microplates were coated with 0.5 μg of NH_2-PEG_{10k}-NH_2 per well overnight in coating buffer (0.05 M Na_2CO_3/0.05 M $NaHCO_3$; pH 9.0), then blocked with 5% skim milk in PBS. Human serum was diluted 25-fold in 2% skim milk in PBS; then 2-fold serial dilutions were made in 4% human serum and 2% skim milk dissolved in PBS. Anti-PEG antibodies were detected with HRP-conjugated secondary antibodies (Supplemental Table 3) and addition of ABTS/H_2O_2. Absorbance was read on a microplate reader at 405 nm. Samples were considered positive for anti-PEG antibodies if their OD readings were three times higher than background, the calculated concentrations were greater than 0.2 μg/mL for IgM and 0.3 μg/mL for IgG, and the signal was reduced by at least 35% in a competitive ELISA.[14] All subsequent experiments involving the use of human serum used pooled serum from subjects who tested negative for anti-PEG antibodies in this ELISA.

Drug Release Assays. Anti-PEG antibodies were serially diluted 2-fold from 100 μg/mL in $GHBS^{+2}$ buffer[41] (0.2% gelatin, 10 mM HEPES, 290 mM NaCl containing 0.3 mM $CaCl_2$, 0.1 mM $MgCl_2$; pH 7.3) in a black Nunc 96-well polypropylene plate (Thermo Fisher Scientific). An equal volume (50 μL) of human serum containing 20 μg/mL PLD (based on doxorubicin content) was added to the anti-PEG antibodies so that the final concentration of doxorubicin was 10 μg/mL and the highest antibody concentration was 50 μg/mL. The plates were covered with plastic film and aluminum foil and incubated for 30 min in a dark 37 °C room on an orbital shaker. Doxorubicin release from liposomes was detected by measuring fluorescence on a Tecan Infinite M1000 Pro at 490/590 nm. One-hundred percent release was determined by replacing antibody with an equal amount of 1% Triton X-100 in PBS. Zero percent release was determined by incubating 10 μg/mL of PLD diluted in $GHBS^{+2}$. Triton X-100 does not interfere with the fluorescence reading of doxorubicin (Supplemental Figure 12). Drug release percentage is calculated as (sample fluorescence − 0% lysis fluorescence) ÷ (100% lysis fluorescence − 0% lysis fluorescence) × 100. Non-PEG-binding human, rat, and mouse IgG and IgM isotype-matched antibodies were included as negative controls.

Complement Inactivation. Serum was treated in two ways to attenuate complement activity. To deplete complement proteins, CVF (Quidel; A600) was added to serum at 20 units per mL and incubated at 37 °C for 90 min.[79] Complement proteins were also inactivated by heating serum to 56 °C for 30 min,[38] then cooled on ice.

Complement Pathway Experiments. Calcium and magnesium ions were depleted from human sera by adding 10 mM EDTA or EGTA, respectively, to 100% human sera and standing the sera at room temperature for 30 min. Ion-chelated sera were added to a mixture of 20 μg/mL Doxisome and 100 μg/mL anti-PEG antibody diluted in GHBS (no supplementation with magnesium or chloride ions) and incubated at 37 °C for 15 min. $MgCl_2$ (20 mM) was added back to serum containing 10 mM EGTA after 15 min to selectively restore the alternative pathway. Drug release was measured *via* fluorescence as described.

A 50 μL amount of complement-depleted sera was incubated with 50 μL of $GHBS^{+2}$ buffer containing 20 μg/mL Doxisome and 100 μg/mL anti-PEG antibodies, then incubated for 15 min at 37 °C. Drug release was measured by fluorescence as described. Purified complement proteins were restored to their respective complement-depleted sera to physiological concentrations (C1q: 100 μg/mL; factor B: 200 μg/mL), then returned to incubation at 37 °C for another 15 min. Drug release was assayed again *via* fluorescence detection.

Cryo-electron Microscopy. Doxisome was incubated for 15 min at 37 °C under different serum and antibody conditions diluted in PBS. Here, we used PBS instead of $GHBS^{+2}$ because gelatin forms white aggregates upon exposure to low temperatures that are required for cryo-EM imaging; however, drug release still occurs under PBS conditions (Supplemental Figure 13).

Samples were prepared for cryo-EM on a FEI Vitrobot by pipetting 4 μL of the reaction mixture onto 200 mesh holey carbon film (Electron Microscopy Sciences), blotted for 3 s, and stored in liquid nitrogen until imaging. Images were taken at 50 000-fold magnification on a FEI Tecnai F20 at a dose of 2528 e/nm^2 s, operating at 200 kV.

***In Vivo* Drug Release.** Male Wistar rats were i.v. injected with 6.4 or 12.8 mg/kg r33G rat anti-PEG IgG or control rat IgG to achieve serum antibody concentrations of approximately 130 or 260 μg/mL as measured by ELISA, respectively. The rats were i.v. injected 30 min later with 2.5, 5, or 10 mg/kg of LC-101 PEGylated liposomal doxorubicin. Rats were anesthetized by isoflurane inhalation, and blood was withdrawn by cardiac puncture into K2-EDTA tubes within 5 min of receiving LC-101. Samples were immediately centrifuged for 12 min at 1500*g* at 4 °C, and plasma was transferred into a clean polypropylene tube.

Released doxorubicin in plasma samples was measured by modification of a previously published method.[54,55] Dowex-50-hydrogen, strongly acidic cation ion-exchange resin, was converted to the sodium form by successive washes with 2 M NaOH, 1 M NaCl, and 0.9% NaCl. Dowex can strongly bind doxorubicin but does not bind negatively charged PLD (Supplemental Figure 15). Based on cryo EM data showing binary release of doxorubicin sulfate from liposomes, we assumed that PLD were either intact or empty. Plasma samples were serially diluted with PBS, and 600 μL aliquots were

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either untreated (leaving total intact PLD plus released doxorubicin) or treated with 100 mg of dried Dowex to remove free doxorubicin (leaving just intact PLD). Plasma was gently mixed for 20 min and centrifuged at 1500*g* for 1 min, and then 100 μL of each plasma sample was transferred to a black fluorescence microtiter plate into wells containing 100 μL of PBS. Doxorubicin fluorescence was measured on a Tecan Infinite M1000 Pro at 490/590 nm.

The percentage of free doxorubicin (DOX) in comparison to total doxorubicin (DOX plus PLD) was estimated from fluorescence measurements of untreated (F) and Dowex-treated (FD) plasma samples. The difference F − FD represents the fluorescence of free doxorubicin in plasma samples. Fluorescence readings increased in plasma samples from rats receiving control antibody after Dowex treatment due to absorption of water by Dowex, which resulted in an increase in the concentration of LC-101. This volume change was used to correct the fluorescence of Dowex-treated plasma samples from rats treated with r33G, calculated as $FD_{r33G}' = FD_{r33G} \times F_C/FD_C$, where FD_{r33G}' is the corrected fluorescence of Dowex-treated plasma samples from rats treated with r33G, FD_{r33G} is the measured fluorescence of Dowex-treated plasma samples from rats treated with r33G, F_C is the measured fluorescence of plasma samples from rats treated with control IgG, and FD_C is the measured fluorescence of Dowex-treated plasma samples from rats treated with control IgG. The fluorescence contribution from LC-101 was estimated to be 12-fold less than free doxorubicin (Supplemental Figure 14E). The percentage of Dox relative to Dox + PLD was then estimated by the formula $100 \times (F_{r33G} - FD_{r33G}')/[(F_{r33G} - FD_{r33G}') + (12FD_{r33G}')]$.

Statistical Analysis. All statistical analyses were performed by GraphPad Prism 7.0 (La Jolla CA, USA, www.graphpad.com). Significance between different treatment groups was analyzed using Student's *t* test. Significant differences in the number of empty liposomes generated by different treatments were estimated by the *z* score test for two population proportions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.9b07218.

Drug release in mouse serum; correlation between anti-PEG IgG antibody binding avidity and Doxisome lytic activity; complement-dependence of anti-PEG antibody-induced drug release of DOX from Doxisome; comparison of DOX release from Doxisome and Doxil; comparison of drug release from Doxisome and Lipo-Dox; drug release by pre-existing human anti-PEG IgG from a normal human donor; control cryo-EM images of Doxisome; wide-view cryo-EM images of Doxisome incubated under various conditions; proportion of empty liposomes under various conditions; DOX release from liposomes by different subclasses of humanized anti-PEG IgG; rate of DOX release from liposomes; influence of Triton X-100 on DOX fluorescence; drug release in PBS; release of DOX from PLD in rats; validation of the Dowex assay; list of anti-PEG antibodies used in this study; relative binding of anti-PEG antibodies to Doxisome; comparison of PLD used in this study; list of commercial antibodies used in this study (PDF)

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Notes

The authors declare the following competing financial interest(s): Yechezkel Barenholz is one of the inventors on two already expired (March 2010) patents relevant to Doxil: (1) Barenholz, Y., and Haran, G. Method of Amphipathic Drug Loading in Liposomes by pH Gradient. U.S. Patent 5,192,549, March 9, 1993. U.S. Patent 5,244,574, September 14, 1993; (2) Barenholz Y., and Haran, G. Liposomes: Efficient Loading and Controlled Release of Amphipathic Molecules, U.S. Patent 5,316,771, May 31, 1994. The Hebrew University received royalties from Doxil sales until the patents expired. Yechezkel Barenholz is also an inventor on another relevant patent: Yechezkel Barenholz, Janos Szebenei, Miklos Toth and Laszlo Rosivall: Particular drug carriers as desensitizing agents U.S. Patent 9,078,812 B2, July 13, 2015. This patent is not yet licensed.

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SUPPORTING INFORMATION

Premature Drug Release from Polyethylene Glycol (PEG)-Coated Liposomal Doxorubicin *via* Formation of the Membrane Attack Complex

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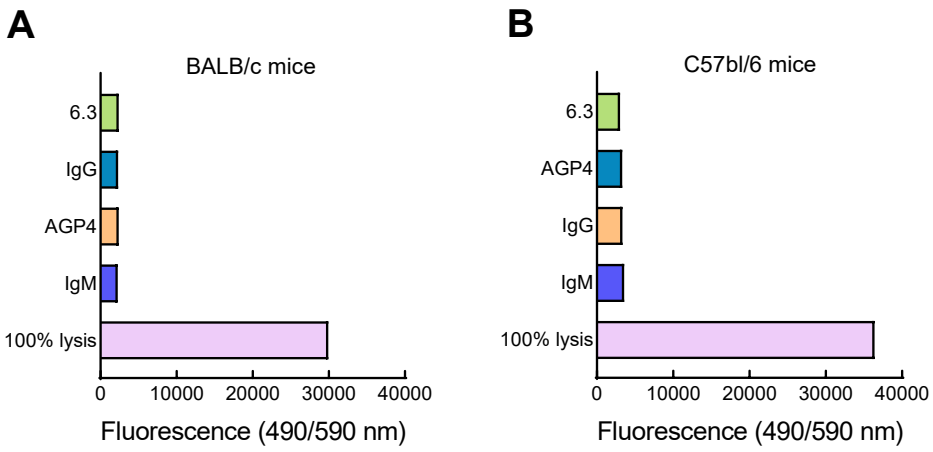
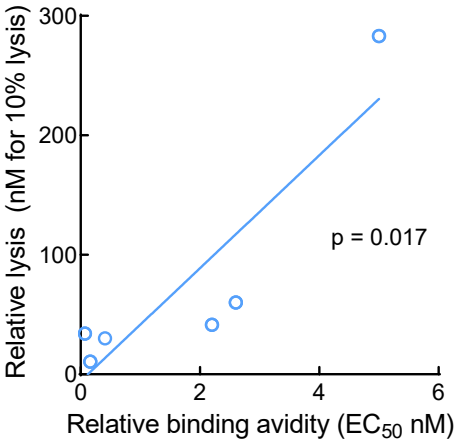


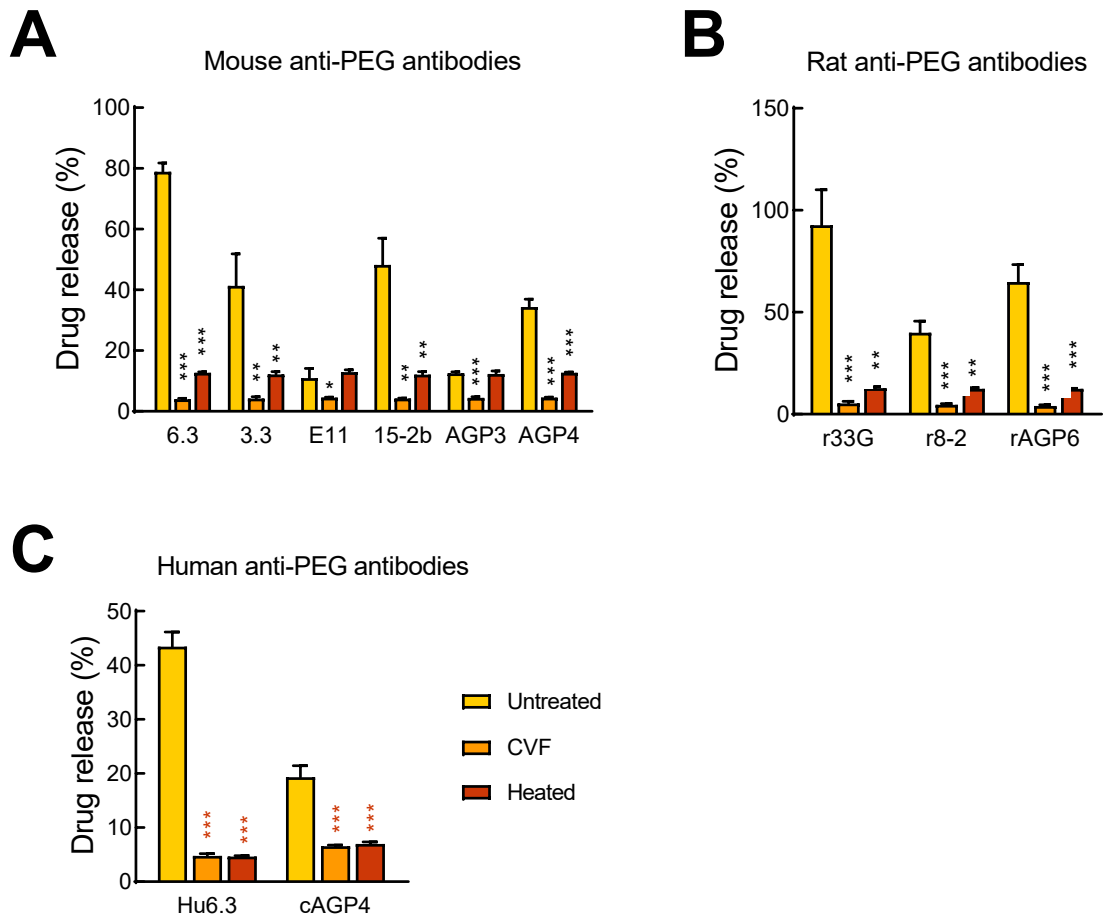
Supplemental Figure 1. Drug release in mouse serum

6.3 (mouse anti-PEG IgG_1) and AGP4 (mouse anti-PEG IgM) were mixed with serum from BALB/c **(A)** or C57bl/6 **(B)** mice containing Doxisome to give a final concentration of 27.5 µg/mL of 6.3, 93 µg/mL of AGP4 and 10 µg/mL of Doxisome, respectively. The same quantity of non-binding IgG_1 and IgM were used as negative controls. 100% lysis was determined by adding Triton X-100 to mouse serum and Doxisome instead of antibodies. Drug release is plotted as fluorescence.



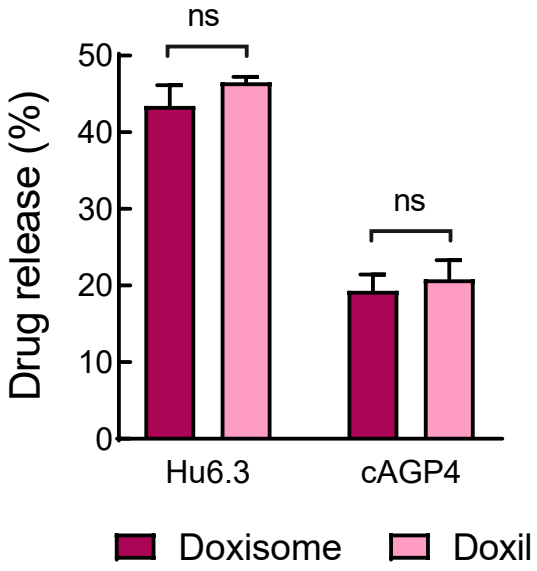
Supplemental Figure 2. Correlation between anti-PEG IgG antibody binding avidity and Doxisome lytic activity

The concentration of anti-PEG IgG antibody that lysed 10% of Doxisome was plotted against the binding EC_{50} concentrations of the anti-PEG antibodies. Antibodies with greater binding avidity (lower EC_{50}) could lyse Doxisome at lower concentrations.



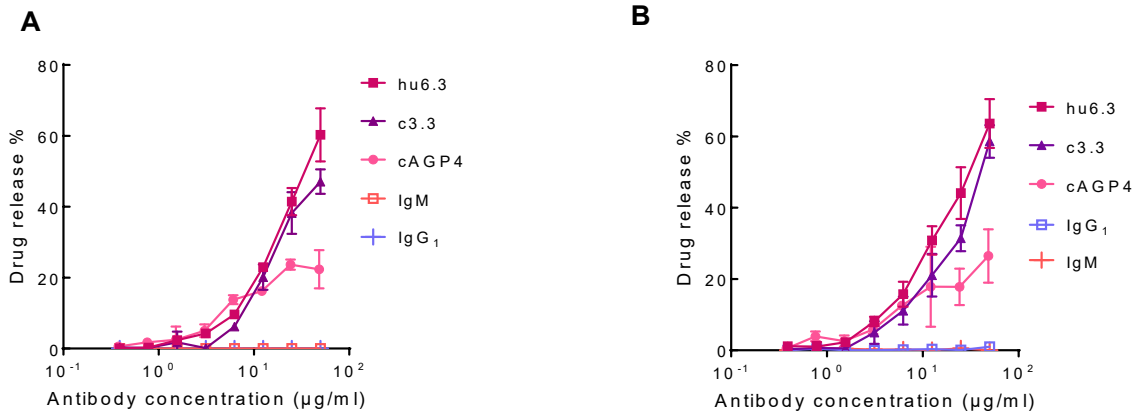
Supplemental Figure 3. Anti-PEG antibody-induced drug release of doxorubicin from Doxisome is complement-dependent

50 µg/mL of mouse anti-PEG (A), rat anti-PEG (B) or humanized anti-PEG (C) antibodies were incubated with 10 µg/mL Doxisome in the presence untreated sera (yellow bars), CVF-treated sera (orange bars) or sera heated to 56 °C (red bars). Error bars show standard deviation, n = 3. Significant differences between untreated serum and CVF or heat treated serum are indicated: *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$.



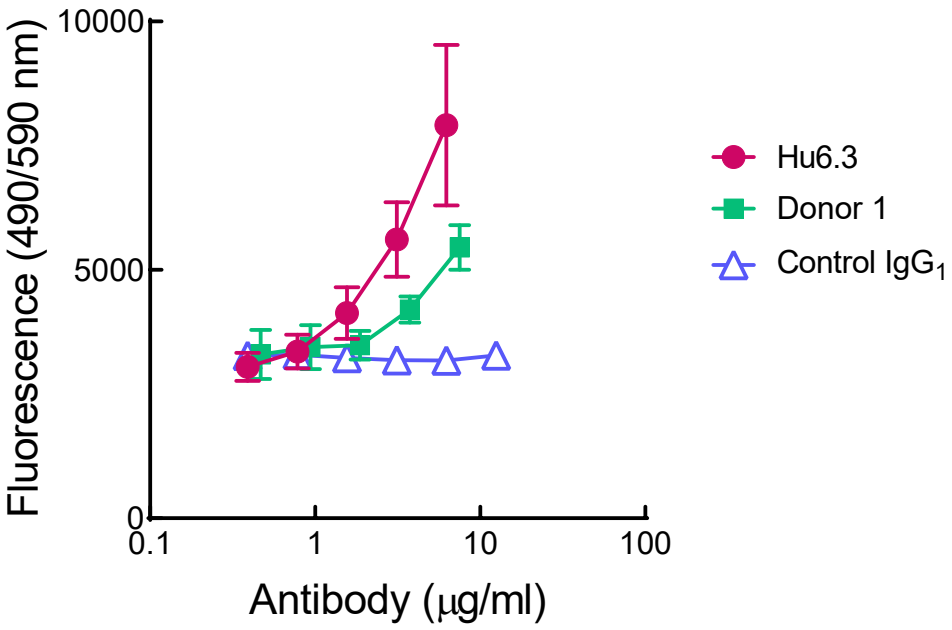
Supplemental Figure 4. Release of doxorubicin from Doxisome and Doxil

Comparison of drug release induced by 50 µg/mL of humanized anti-PEG IgG and IgM incubated in human serum and Doxisome (red) or Doxil (pink). Percentage drug release was calculated in comparison to 100% drug lysis with Triton X-100, and 0% drug lysis with PBS. Results show mean values ± SD, n = 3. Significance is reported as $p > 0.01$ (ns).



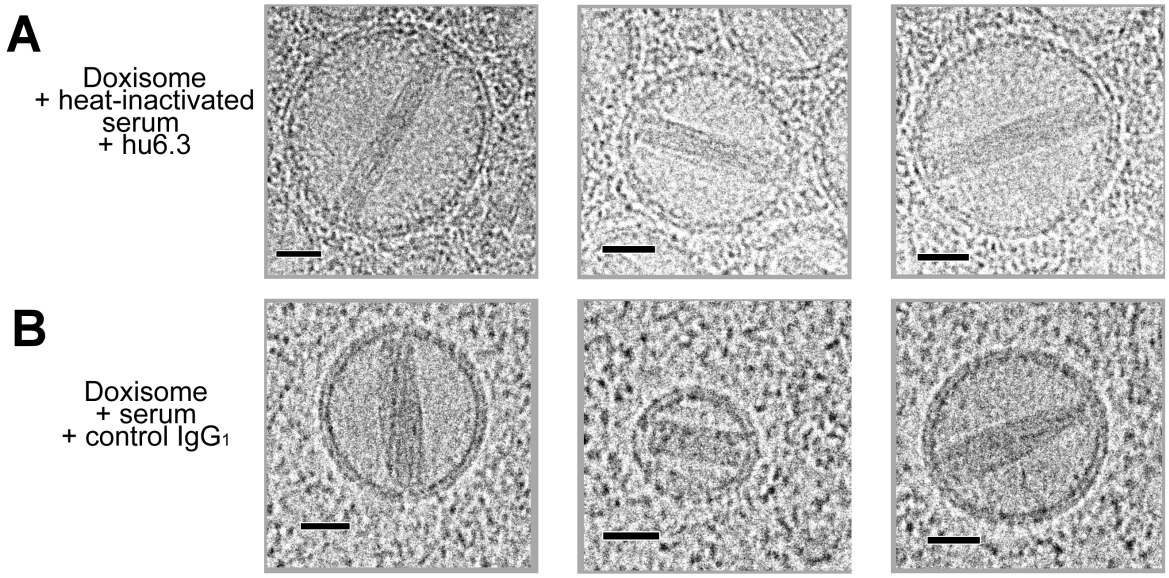
Supplemental Figure 5. Comparison of drug release from Doxisome and Lipo-Dox

Humanized anti-PEG antibodies were serially diluted in PBS and human serum containing 20 µg/mL of **(A)** Doxisome or **(B)** Lipo-Dox were added to the dilutions. Mixtures were incubated for 30 min before fluorescence of released doxorubicin was measured. Bars show standard deviation, n = 3.



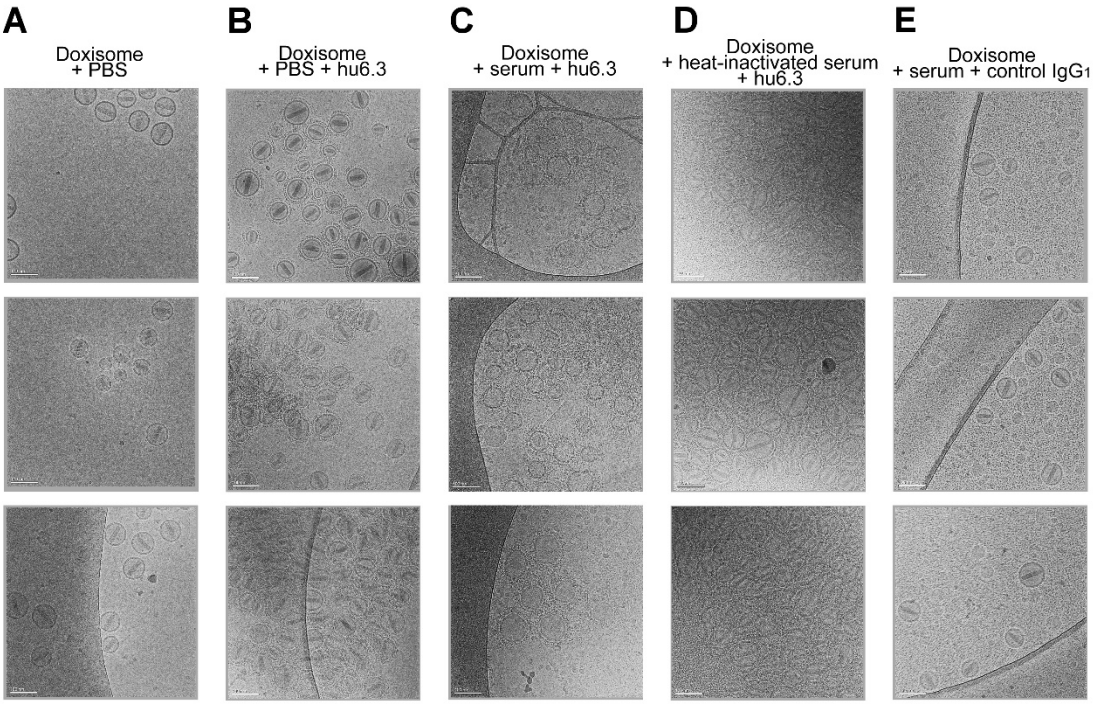
Supplemental Figure 6. Drug release by pre-existing human anti-PEG IgG

50 µl of human serum containing 15 µg/mL of anti-PEG IgG (Donor 1) was serially diluted two-fold, then added to an equal volume of human serum without anti-PEG antibody containing 20 µg/mL Doxisome. Hu6.3 and control IgG_1 antibody were used as positive and negative controls, respectively. Drug release is plotted as fluorescence. Error bars show standard deviation, n = 8.



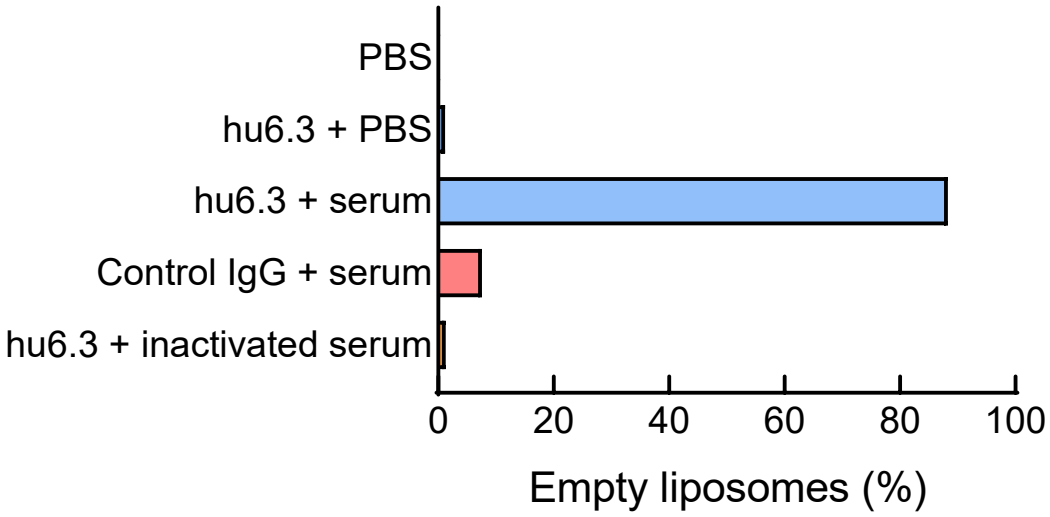
Supplemental Figure 7. Control cryo-EM images of Doxisome.

A) Doxisome incubated with hu6.3 and heat-inactivated serum. **B)** Doxisome incubated with control IgG_1 in human serum. Scale bars represent 25 nm.



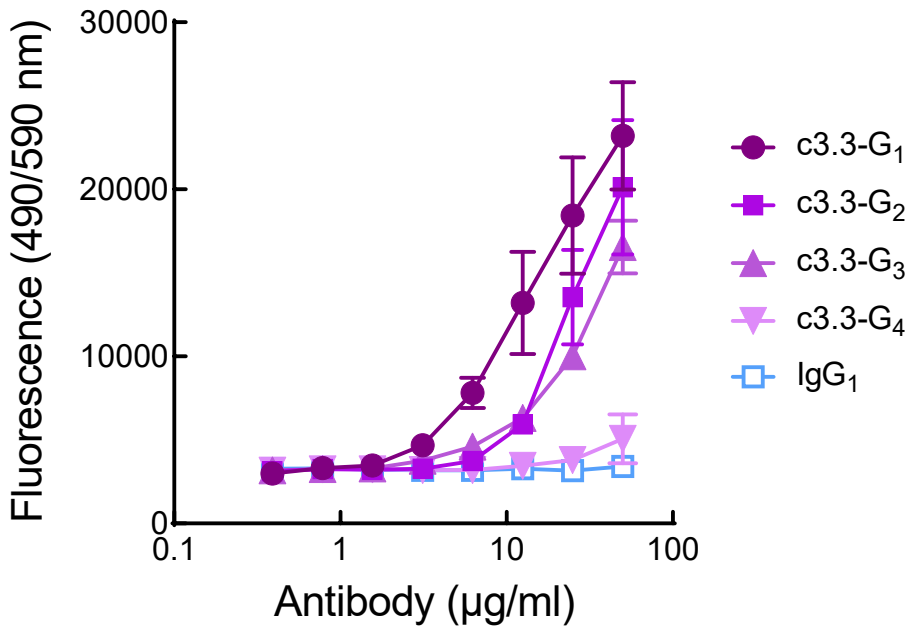
Supplemental Figure 8. Wide-view cryo-EM images of Doxisome incubated under various conditions.

A) Doxisome in PBS. **B)** Doxisome in PBS and hu6.3. **C)** Doxisome in human serum and hu6.3. Reaction mixtures of Doxisome/human serum/hu6.3 were passed through a Sephadex CL-4B column to remove free antibody and observe C5b-9 complexes binding to Doxisome. **D)** Doxisome incubated with hu6.3 and heat-inactivated serum. **E)** Doxisome in human serum and control IgG_1. Scale bars represent 100 nm.



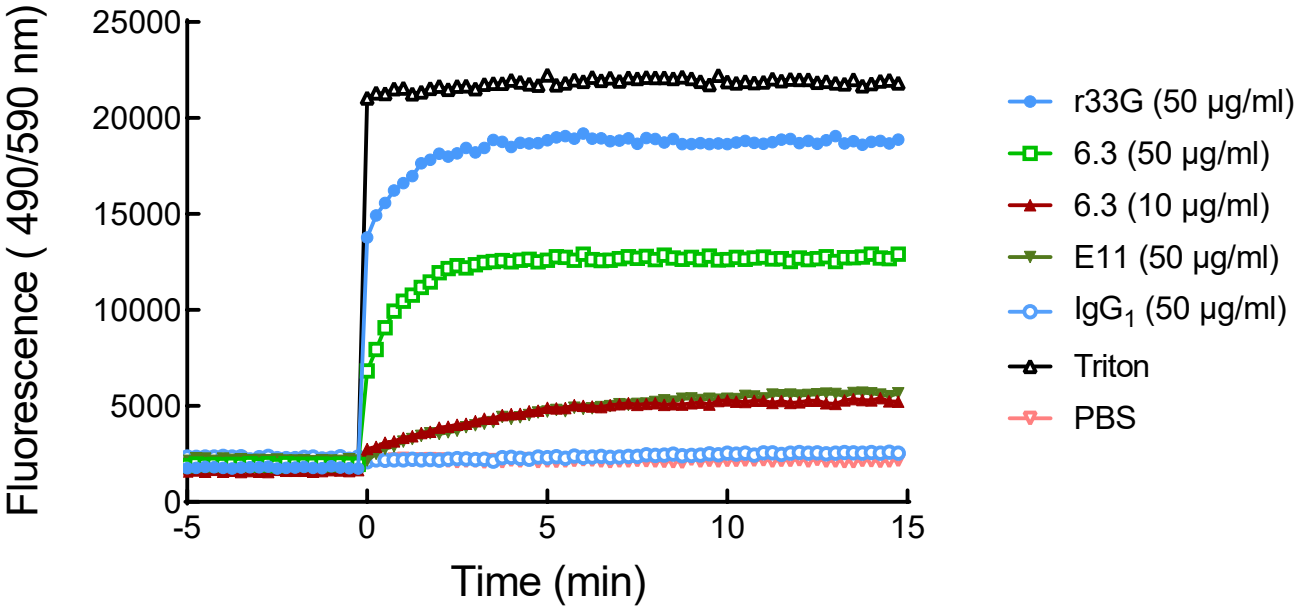
Supplemental Figure 9. Proportion of empty liposomes

Wide-view cryo-EM pictures were analysed by counting the number of liposomes with and without visible Doxorubicin nanorods present. Empty liposomes are expressed as a percentage of total liposomes.



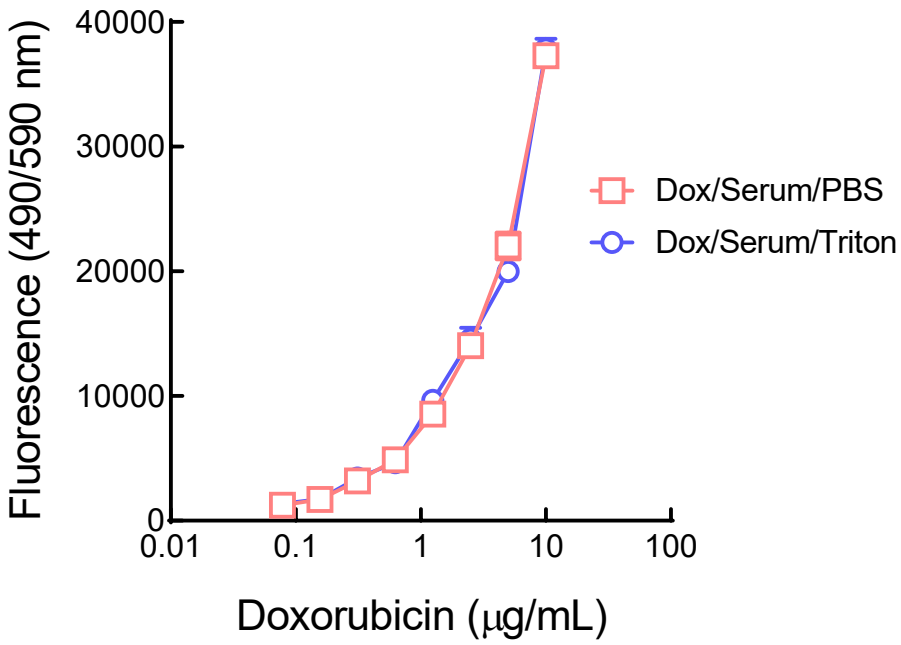
Supplemental Figure 10. Doxorubicin release from liposomes by different subclasses of humanized anti-PEG IgG.

The ability of a series humanized anti-PEG IgG subclasses to cause drug release from Doxisome was measured as described in methods and materials. Results show mean values ± SD, n = 8.



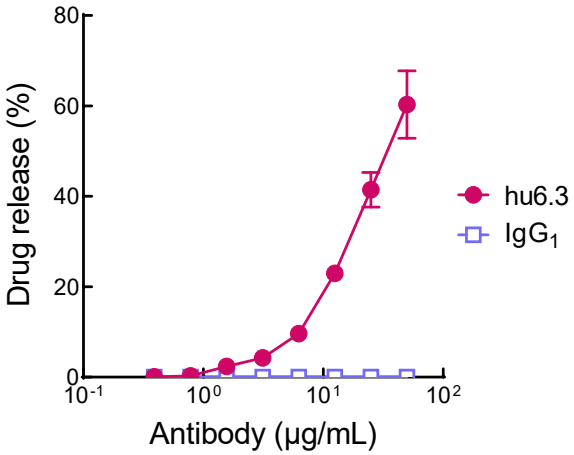
Supplemental Figure 11. Rate of doxorubicin release from liposomes

Doxisome was mixed to 20 µg/mL in Wistar rat serum and added to a black 96-well plate. The plate's fluorescence was detected at 490/590nm with a microplate reader (Tecan Infinite® M1000 Pro) every 15 seconds for five minutes at 37 ˚C. At time zero, an equal volume of 100 µg/mL r33G, 6.3 or E11, or 20 µg/mL 6.3 was added to the wells in triplicate. As controls, anti-PEG antibodies were replaced with either 100 µg/mL control IgG_1, PBS or 1% Triton X-100 in PBS and the fluorescence was recorded for another 15 minutes.



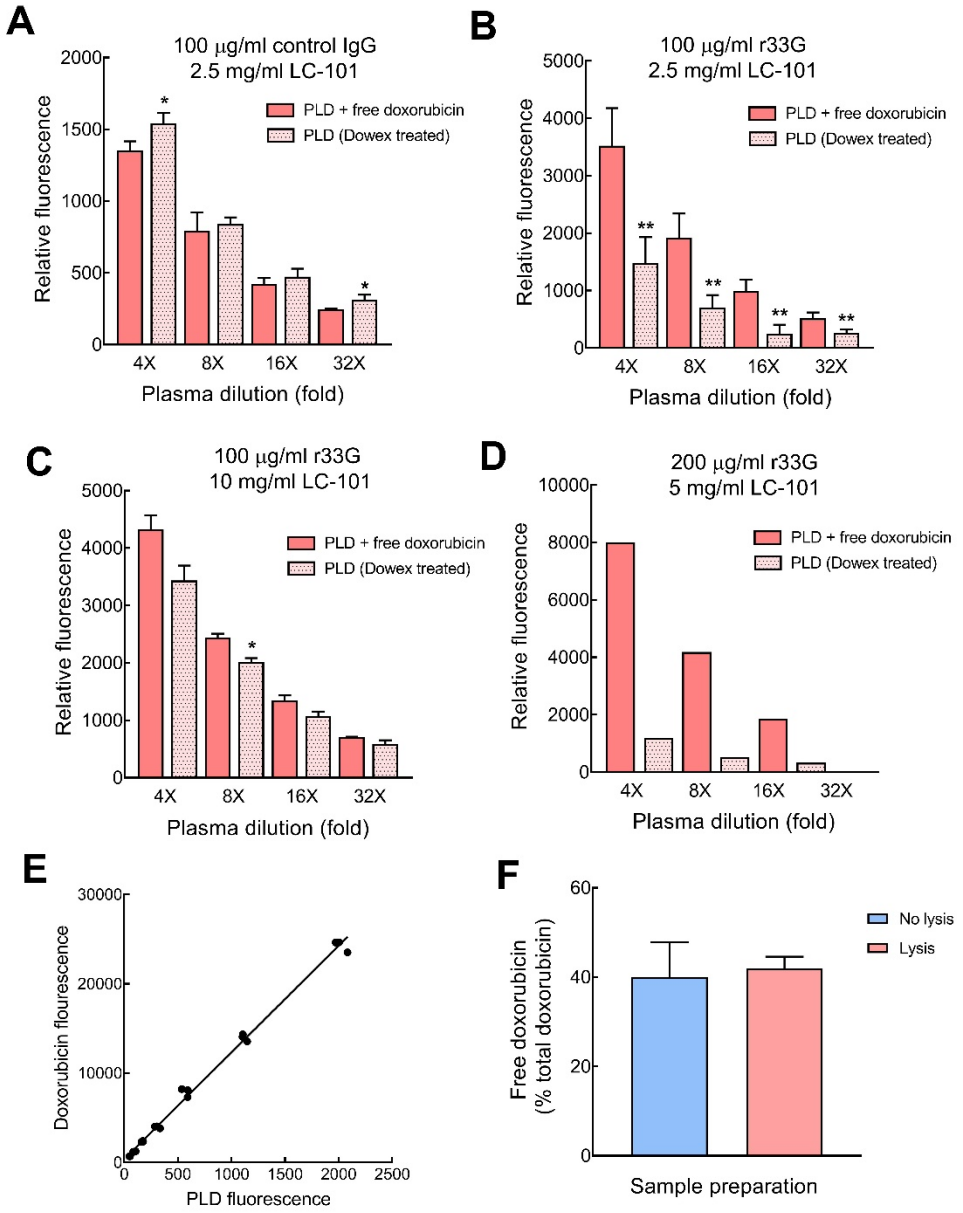
Supplemental Figure 12. Influence of Triton X-100 on doxorubicin fluorescence

Rat serum containing 20 μg/mL of doxorubicin was serially diluted two-fold in rat serum in a 96-well black plate. An equal volume of PBS or 1% Triton X-100 was added, and the fluorescence of the wells was measured. Error bars show standard deviation, n = 3.



Supplemental Figure 13. Drug release in PBS

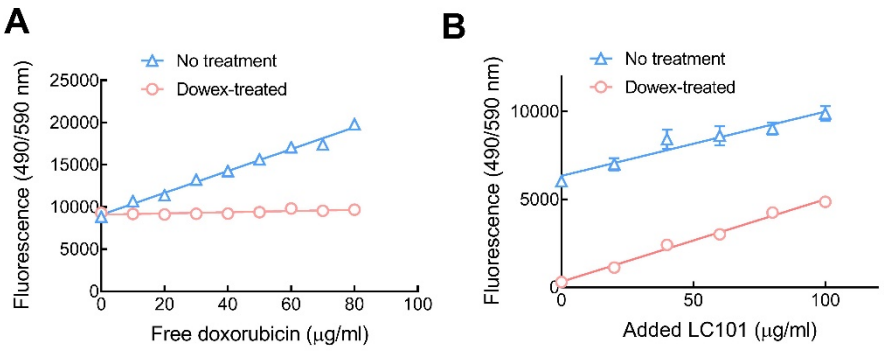
For cryo-EM images, antibodies were diluted in PBS rather than $GHBS^{++}$. We show here that drug release can still be detected under this condition. Bars show standard deviation, n =3.



Supplemental Figure 14. Release of doxorubicin from PLD in rats

A-D) Male Wistar rats were i.v. injected with a control rat IgG or r33G rat anti-PEG IgG to achieve the indicated serum concentrations of antibodies. After 1 h, the rats were i.v. injected with the indicated doses of LC-101 PLD. Plasma samples obtained from the rats within 5 min of LC-101 injection were serially diluted in PBS and the fluorescence of either untreated plasma (solid columns) or Dowex-treated plasma (dotted columns) was measured. **E**) The fluorescence of free doxorubicin versus LC-101 fluorescence. The concentrations of doxorubicin in PLD and free doxorubicin were identical at each point. The slope of the line (12.0) corresponds to the ratio of free doxorubicin fluorescence as compared to LC-101 fluorescence. **F**) Comparison of the amount of free doxorubicin in plasma of rats treated as in panel D by two methods. The first method uses plasma treated with or without Dowex as

described in materials and methods (no lysis). The second method uses plasma treated with or without Dowex but then lyses LC-101 in all samples with 1% Triton X-100 to measure total doxorubicin in the samples (Lysis). Comparison of the calculated doxorubicin release by the two methods was not significantly different, indicating good estimation of doxorubicin release.



Supplemental Figure 15. Validation of the Dowex assay

A) A constant amount of LC-101 PLD (50 µg) in 800 µL of 25% rat serum/PBS was spiked with the indicated concentrations of free doxorubicin. The samples were untreated or treated to remove free doxorubicin by addition of 100 mg Dowex resin for 20 min at 4ºC. The fluorescence of the samples in 96-well black fluorescence plates was measured on a fluorescence reader. Dowex treatment removed free doxorubicin so only the fluorescence of the remaining LC-101 PLD is observed. **B**) The indicated concentrations of LC-101 was added to 600 µL of 25% rat serum/PBS containing a constant amount of free doxorubicin (1 µg/mL). The samples were untreated or treated to remove free doxorubicin by addition of 100 mg Dowex resin for 20 min at 4ºC. The fluorescence of the samples in 96-well black fluorescence plates was measured on a fluorescence reader. The continually increasing fluorescence is due to the presence of LC-101, which is not adsorbed by Dowex resin.

Supplemental Table 1. List of anti-PEG antibodies

Antibody name	Fc region	Subclass	Epitope
6.3	Mouse	IgG_1	PEG backbone
3.3	Mouse	IgG_1	PEG backbone
E11	Mouse	IgG_1	PEG backbone
15-2b	Mouse	IgG_{2b}	Methoxy group
AGP3	Mouse	IgM	PEG backbone
AGP4	Mouse	IgM	PEG backbone
Hu6.3	Human	IgG_1	PEG backbone
c3.3-G_1	Human	IgG_1	PEG backbone
c3.3-G_2	Human	IgG_2	PEG backbone
c3.3-G_3	Human	IgG_3	PEG backbone
c3.3-G_4	Human	IgG_4	PEG backbone
cAGP4	Human	IgM	PEG backbone
r8-2	Rat	IgG_{2a}	PEG backbone
r33G	Rat	IgG_{2a}	PEG backbone
rAGP6	Rat	IgM	PEG backbone

Supplemental Table 2. Relative binding of anti-PEG antibodies to Doxisome. Results show the concentrations of antibody producing 50% maximal signal when binding to Doxisome in ELISA.

Antibody	Class	Species	Binding EC_{50} (nM)
15-2b	IgG	mouse	0.07
6.3	IgG	mouse	0.41
3.3	IgG	mouse	2.2
E11	IgG	mouse	5.0
AGP3	IgM	mouse	0.035
AGP4	IgM	mouse	0.042
r33G	IgG	rat	0.16
r8-2	IgG	rat	2.6
rAGP6	IgM	rat	0.053

Supplemental Table 3. Comparison of PLD used in this study

Formulation	**Doxil**	**Doxisome**	**Lipo-Dox**	**LC-101**
PEG-lipid (molar %)	5%			
Diameter	~90 nm			
Doxorubicin concentration	2 mg/ml			
Lipid	HSPC	HSPC	DSPC	HSPC
Lipid (molar %)	56%	56%	56%	56%
Cholesterol (molar %)	39%	39%	39%	39%
Manufacturer (country)	Johnson & Johnson (USA)	Taiwan Liposome Company (Taiwan)	TTY Biopharm (Taiwan)	Lipocue (Israel)

Supplemental Table 4. List of commercial antibodies

Antibody	Catalogue no.	Supplier
HRP-goat anti-human IgG	109-036-098	Jackson Immunoresearch
HRP-goat anti-human IgM	109-036-129	Jackson Immunoresearch
HRP-donkey anti-mouse IgG	715-035-150	Jackson Immunoresearch
HRP-goat anti-mouse IgM	115-035-020	Jackson Immunoresearch
HRP-goat anti-rat IgG	112-035-003	Jackson Immunoresearch
HRP-goat anti-rat IgM	112-035-075	Jackson Immunoresearch
Human IgG_1	ab90283	Abcam
Human IgM	009-000-012	Jackson Immunoresearch