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REVIEW

Polyethylene Glycol Immunogenicity: Theoretical, Clinical, and Practical Aspects of Anti-Polyethylene Glycol Antibodies

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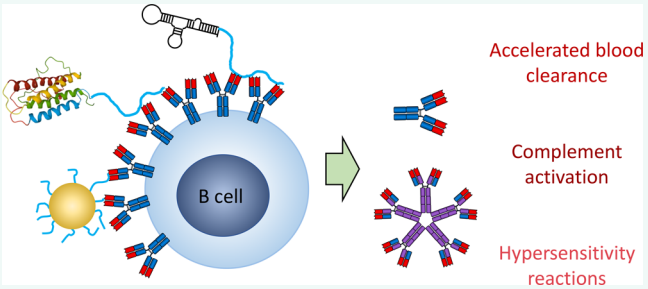
ABSTRACT: Polyethylene glycol (PEG) is a flexible, hydrophilic simple polymer that is physically attached to peptides, proteins, nucleic acids, liposomes, and nanoparticles to reduce renal clearance, block antibody and protein binding sites, and enhance the half-life and efficacy of therapeutic molecules. Some naïve individuals have pre-existing antibodies that can bind to PEG, and some PEG-modified compounds induce additional antibodies against PEG, which can adversely impact drug efficacy and safety. Here we provide a framework to better understand PEG immunogenicity and how antibodies against PEG affect pegylated drug and nanoparticles. Analysis of published studies reveals rules for predicting accelerated blood clearance of pegylated medicine and therapeutic liposomes. Experimental studies of anti-PEG antibody binding to different forms, sizes, and immobilization states of PEG are also provided. The widespread use of SARS-CoV-2 RNA vaccines that incorporate PEG in lipid nanoparticles make understanding possible effects of anti-PEG antibodies on pegylated medicines even more critical.



KEYWORDS: *polyethylene glycol, immunogenicity, anti-PEG antibodies, pre-existing antibodies, thymus-independent type-2 (TI-2) antigen, SARS-CoV-2 RNA vaccines, pegylation, accelerated blood clearance, liposomes, humoral immunity*

Attachment of polyethylene glycol (PEG) to small molecules, nucleotides, peptides, proteins, liposomes, and nanoparticles is widely used to improve their stability, solubility, and pharmacokinetic properties.[1,2] Increased half-life in the circulation is particularly advantageous for injectable drugs because administration frequency can be reduced, leading to better patient compliance and quality of life. Due to the beneficial properties of PEG, a range of pegylated protein (Table 1) and non-protein (Table 2) medicines are clinically available, including RNA vaccines against SARS-CoV-2.[3,4]

The size and number of PEG molecules attached to a compound can be varied depending on the desired purpose. A single linear or branched methoxy PEG (mPEG) molecule ranging in size from 12 to 60 kDa is attached to peptides, nucleotides, and small recombinant proteins to increase their hydrodynamic diameter, thereby reducing uptake by the kidney. On the other hand, multiple $mPEG_{5000}$ molecules are attached to the surface of foreign enzymes to increase *in vivo* stability and block binding of anti-enzyme antibodies. Hundreds or even thousands of $mPEG_{2000}$−lipid molecules are incorporated in liposomes and nanoparticles to reduce uptake by resident macrophages in the liver. Although PEG is typically depicted as a small linear molecule with dimensions on the order of a small protein, the actual contour lengths of commonly used PEG molecules range from 12.5 nm for PEG_{2000} to 253 nm for linear $PEG_{40,000}$ (Table 3). A more realistic illustration of common formats of pegylated therapeutics is shown in Figure 1.

Administration of some pegylated drugs results in the generation of antibodies that specifically bind to PEG and reduce treatment efficacy or cause adverse drug reactions.[5−8] The increasing realization that anti-PEG antibodies may have clinical impact is reflected in the United States Food and Drug Administration calling for measurement of anti-PEG antibody responses in new drugs that incorporate PEG molecules.[9]

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Table 1. Clinically Used Pegylated Protein Drugs

brand name	common name	component	source	type	PEG (kDa)	PEG number	disease	year approved
Adagen	pegademase	adenosine deaminase	bovine	enzyme	5	11−17	severe combined immunodeficiency	1990
Oncaspar	pegaspargase	L-asparaginase	*E. coli*	enzyme	5	69−82	leukemia	1994
PEG-Intron	PEG interferon	interferon alfa-2b	human	cytokine	12	1	hepatitis C	2001
Neulasta	pegfilgrastim	G-CSF	human	cytokine	20	1	neutropenia	2002
Pegasys	peginterferon alfa-2a	interferon alfa-2b	human	cytokine	40	1 (branched)	hepatitis	2002
Somavert	pegvisomant	antagonist (GHR)	human	protein	5	4−6	acromegaly	2003
Mircera	PEG-epoetin beta	epoetin beta	human	protein	30	1	anemia	2007
Cimzia	certolizumabpegol	anti-TNFa Fab	human	antibody	40	1 (branched)	rheumatoid arthritis	2008
Krystexxa	pegloticase	uricase	porcine	enzyme	10	9	gout	2010
Sylatron	peginterferon alfa-2b	interferon alfa-2b	human	cytokine	12	1	melanoma	2011
Lonquez	lipegfilgrastim	G-CSF	human	cytokine	20	1	neutropenia	2013
Plegridy	peginterferon beta-1a	interferon beta-1a	human	cytokine	20	1	multiple sclerosis	2014
Adynovate	PEG-antihemophilic factor	factor VIII	human	protein	20	1 (branched)	hemophilia A	2015
Rebinyn	coagulation factor IX	factor IX	human	protein	40	1	hemophilia B	2017
Jivi	PEG-antihemophilic factor	factor VIII	human	protein	60	1 (branched)	hemophilia A	2018
Fulphilia	pegfilgrastim	G-CSF	human	cytokine	20	1	neutropenia	2018
Revcovi	elapegademase	adenosine deaminase	bovine	enzyme	5	13	severe combined immunodeficiency	2018
Asparlas	calaspargase pegol	L-asparaginase	*E. coli*	enzyme	5	31−39	leukemia	2018
Palynziq	pegvaliase	lyase	cyanobacteria	enzyme	20	9	phenylketonuria	2018
Esperoct	glycoPEG-antihemophilic factor	factor VIII	human	protein	40	1	hemophilia A	2019
Ziextenzo	pegfilgrastim	G-CSF	human	cytokine	20	1	neutropenia	2019
Udenyca	pegfilgrastim	G-CSF	human	cytokine	20	1	neutropenia	2019

Table 2. Clinically Used Pegylated Non-protein Drugs

brand name	common name	component	source	type	PEG (kDa)	PEG number	disease	year approved
Doxil	pegylated liposomal doxorubicin (PLD)	doxorubicin	lipid	liposome	2	multiple	cancer	1995
Macugen	pegaptanib	anti-VEGF aptamer	nucleotide	nucleotide	40	1 (branched)	macular degeneration	2004
Movantik	naloxegol	antagonist ($C_{34}H_{53}NO_{11}$)	drug	small molecule	0.3	1	constipation	2014
Onivyde	irinotecan liposome	irinotecan	lipid	liposome	2	multiple	cancer	2015
Onpattro	patisiran	siRNA in lipid NP	nucleotide	nanoparticle	2.5	multiple	amyloidosis	2018
Comirnaty	toxinameran (BNT162b2)	mRNA in lipid NP	nucleotide	nanoparticle	2	multiple	COVID-19	2020
Moderna COVID-19 vaccine	mRNA-1273	mRNA in lipid NP	nucleotide	nanoparticle	2	multiple	COVID-19	2020

Many normal individuals also have pre-existing antibodies against PEG in their circulation, likely due to the widespread use of PEG in many cosmetic and healthcare products.[10]

Several excellent reviews cover PEG chemistry,[11,12] assay of pegylated compounds,[13] and immunogenicity of pegylated medicines.[10,14−16] In the present review, we aim to clear up some misconceptions about PEG immunogenicity, realistically assess the impact of anti-PEG antibodies on pegylated medicines, and describe the specificity and binding behavior of anti-PEG antibodies. We survey the literature as well as draw from personal experience over the past 20 years on creating anti-PEG monoclonal antibodies,[17] developing anti-PEG antibody assays,[18−20] assaying and discovering genetic markers for anti-PEG antibodies,[21,22] investigating the effects of anti-PEG antibodies on pegylated medicines,[17,23−25] and creating recombinant anti-PEG receptors and targeting molecules[26−34] to provide a framework to understand what makes a pegylated drugs immunogenic, how anti-PEG antibodies affect the efficacy and safety of pegylated medicines, and some experience with how anti-PEG antibodies behave in practice.

PRE-EXISTING ANTIBODIES TO PEG

Many people who have never taken pegylated medicines have anti-PEG antibodies in their circulation. Early studies using hemagglutination of PEG-modified red blood cells found between 0.2% and 25% of normal donors had antibodies specific to PEG in their plasma.[39,40] Subsequent studies

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Table 3. Dimensions of Commonly Used PEG Molecules

name	MW (Da)	number of ethylene oxide subunits	contour length[a] (nm)	viscosity diameter[b] (nm)
PEG_{2000}	2 000	45	12.5	2.7
PEG_{5000}	5 000	114	31.7	4.5
$PEG_{20\,000}$	20 000	455	126	9.7
$PEG_{30\,000}$	30 000	682	190	12.2
$PEG_{40\,000}$	40 000	909	253	14.3
$PEG_{40\,000}$ branched	40 000	455 × 2	126 × 2	14.3
$PEG_{60\,000}$ branched	60 000	682 × 2	190 × 2	17.9

[a]Ethylene oxide subunit length is taken as 0.278 nm in water.[35] [b]Estimated from $R_h = 0.1912M_r^{0.559}$, where R_h is the viscosity radius of PEG in angstroms, and M_r is the molecular weight of PEG in daltons.[36]

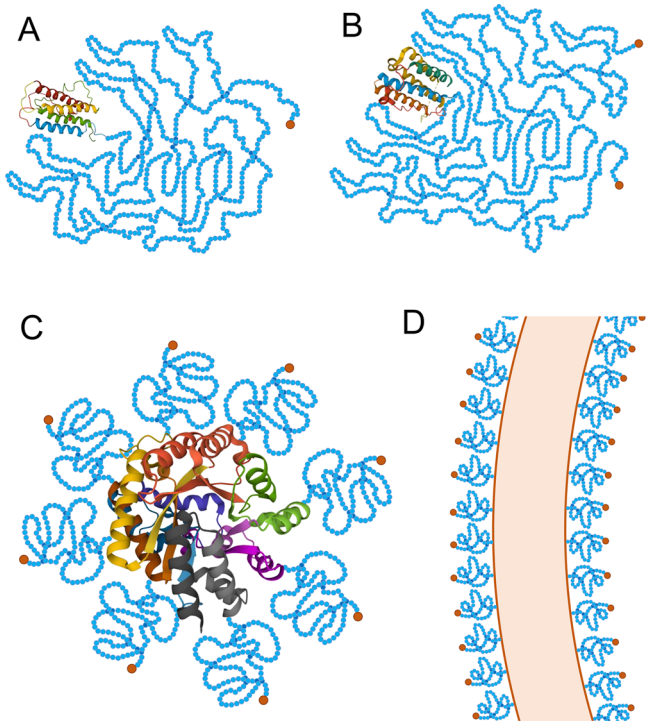

Figure 1. Illustrations of pegylated compounds. (A) $PEG_{30\,000}$ conjugated to a protein such as human erythropoietin. (B) A branched $PEG_{40\,000}$ linked to interferon which has a roughly cylindrical shape with dimensions of approximately 2.0 × 3.0 × 4.0 nm.[37] (C) Eight molecules of PEG_{5000} linked to a protein such as bovine adenosine deaminase. (D) A section of a 100 nM diameter liposome with lipid-PEG_{2000} integrated in the lipid bilayer. Ethylene oxide subunit length is taken to be 0.278 nm.[35] Structure images were illustrated using the PDB files 1BUY, 7E0E, and 1VFL with the Mol* Viewer.[38]

verified the presence of pre-existing anti-PEG antibodies, but a wide range of positive frequencies have been reported (Table 4). Differences in assay formats can explain much of the differences in the reported values. Direct binding assays using beads or ELISA plates coated with PEG derivatives provide high sensitivities, whereas bridging assays underestimate the presence of anti-PEG IgG antibodies. Differences in assay cut-off criteria also impact positive frequencies since many donors have relatively low levels of pre-existing anti-PEG antibodies in their plasma. The notion that the frequency of pre-existing anti-PEG antibodies is increasing over time is probably due to a shift to more sensitive assays.[41]

In mice, a special population of B cells (B-1 cells) spontaneously secrete natural antibodies in the absence of exogenous immunization to provide pre-existing, immediate defense against microbial infections.[42] The existence of analogous human B-1 cells is controversial,[43] but other B cell populations may play a similar role in humans.[44,45] A genome-wide association study found that the presence of pre-existing anti-PEG IgM antibodies is associated with a specific variable segment of the immunoglobulin heavy-chain gene, suggesting that some individuals may have natural antibodies that bind PEG or are more sensitive to casual PEG exposure.[22] Casual exposure to PEG compounds present in pharmaceutical, cosmetic, and health care products may induce anti-PEG antibodies,[46] which may be promoted by inflammatory responses at sites of dermal abrasion and inflammation.[10,47]

Recent studies measuring the prevalence of pre-existing anti-PEG antibodies use human or humanized anti-PEG IgM and IgG antibodies as reference standards, an important step to facilitate comparison of results from different laboratories.[21,41] We confirmed the results of a previous study[21] using humanized anti-PEG IgG and IgM monoclonal antibodies as reference standards to measure the prevalence of anti-PEG antibodies in 1504 healthy Han Chinese donors residing in Taiwan by assaying plasma samples from an additional 900 healthy Han Chinese donors in Taiwan. No significant differences in the frequencies of anti-PEG antibodies was observed between the original 1504 donors[21] and the additional 900 donors (Supplemental Figure 1). A summary of the sex and age distribution of the donors is shown in Supplemental Table 1. Aggregation of the data reveals that of 2404 healthy donors, 634 (26.4%) had anti-PEG IgM antibodies, 601 (25%) were positive for anti-PEG IgG antibodies, and 199 (8.3%) were positive for both anti-PEG IgM and IgG antibodies (Figure 2A). The overall percentage of normal donors that were positive for anti-PEG antibodies (IgG or IgM) was 43.1%. Female donors had higher prevalence of both anti-PEG IgG and IgM antibodies than males (Figure 2B,C). The incidence and concentration of anti-PEG IgM did not significantly vary with age, whereas both the incidence and concentration of anti-PEG IgG deceased with age. Anti-PEG IgM concentrations ranged from 0.2 to 57 $\mu g\ mL^{-1}$, with mean and median concentrations of 1.5 and 0.8 $\mu g\ mL^{-1}$, respectively (Figure 2D). Anti-PEG IgG concentrations ranged from 0.3 to 238 $\mu g\ mL^{-1}$, with mean and median concentrations of 6.2 and 2.1 $\mu g\ mL^{-1}$, respectively (Figure 2E).

ANTI-PEG ANTIBODY RESPONSES TO THYMUS-DEPENDENT ANTIGENS

PEGylated proteins and peptides can elicit anti-PEG antibody responses by the classical T-cell-dependent pathway. Naïve B cells express membrane-bound IgM and IgD immunoglobulins with the same antigen-binding specificity that act as B cell receptors (BCRs).[56] B cells that express BCRs with specificity for PEG are activated when repeating epitopes present in the PEG backbone cross-link multiple BCRs. This can induce differentiation of naïve B cells into plasmablasts that secrete IgM antibodies against PEG. However, a robust IgG antibody response requires that anti-PEG B cells receives additional signals or help from specialized $CD4^+$ T cells called follicular helper T cells (T_{FH} cells) in secondary lymphoid organs.

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Table 4. Comparison of the Prevalence of Anti-PEG Antibodies among Different Studies

year	sample population	sample number	females/ males	anti-PEG antibody positive	anti-PEG IgM positive	anti-PEG IgG positive	both IgG and IgM positive	assay method	ref
1984	naïve donors	453	NR	0.2%	NR	NR		hemagglutination	48
1984	naïve allergy patients	92	NR	3.3%	NR	NR		hemagglutination	48
2004	naïve donors	250	NR	25%	14%	18%		hemagglutination	49
2007	gout patients	24	4/20	NR	NR	8.3%		direct ELISA against 10-kDa mPEG-glycine	50
2011	naïve donors	350	NR	4.3%	NR	NR		bridging assay using hapten-$PEG_{40\,000}$	51
2014	naïve severe gout patients	30	8/22	19%	NR	NR		direct ELISA against 10-kDa mPEG-glycine + competition ELISA	6
2015	naïve acute coronary syndrome patients	354	NR	36%	NR	NR		direct ELISA against 10-kDa mPEG-nitrophenyl carbonate + competition ELISA	52
2015	naïve HBeAg+ subjects	32	NR	6.3%	NR	NR		bridge assay using PEG-IFN or direct ELISA	53
2016	naïve donors	377	151/226	36.8%[a]	31%[a]	8.5%[a]	2.7%[a]	direct ELISA against DSPE-PEG_{5000} + competition ELISA	41
2016	naïve donors	1310		23.5%	13.6%	13.5%		direct ELISA against branched $PEG_{20\,000}$-HSA	54
2020	acute lymphocytic leukemia pediatric patients	673	272/401		29.1%	13.9%		flow cytometry assay of beads coated with PEG_{5000}	55
2016 and 2021	naïve donors	2404	1209/1195	43.1%[b]	26.4%[b]	25.0%[b]	8.3%[b]	direct ELISA against 10-kDa NH_2-PEG-NH_2 + competition ELISA	21 and this report

[a]Cut-off value of 0.1 μg mL^{-1} for both IgG and IgM. [b]Cut-off values of 0.2 μg mL^{-1} for IgG and 0.3 μg mL^{-1} for IgM. NR, not reported.

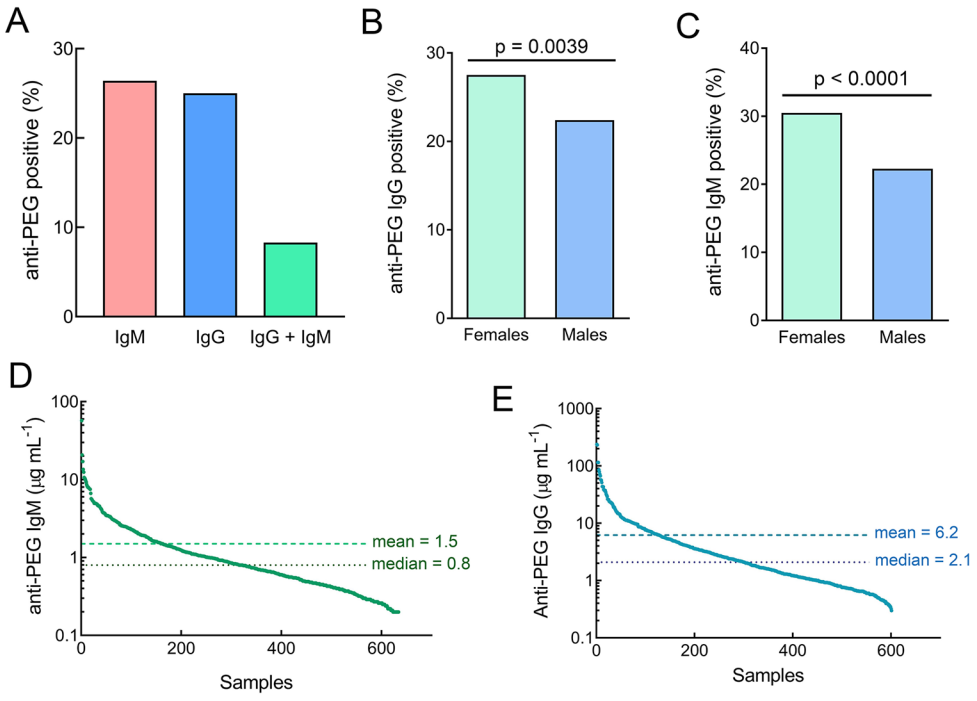

Figure 2. Prevalence of pre-existing human anti-PEG antibodies in healthy donors. (A) Frequencies of donors positive for anti-PEG IgM, IgG, or both IgG and IgM antibodies in 2404 healthy donors. The incidence of anti-PEG IgG (B) and anti-PEG IgM (C) in females and males is shown. The distributions of anti-PEG IgM (n = 634) (D) and anti-PEG IgG (n = 601) (E) concentrations in positive donors are shown. Upper dotted lines indicate the mean antibody concentration while the lower dashed lines indicate the median antibody concentration.

Binding of PEG to multiple BCRs induces internalization and routing of bound PEG-conjugates to internal vesicles where the protein portion of the conjugate is enzymatically digested and some of the resulting peptide fragments are bound by MHC class II molecules for delivery to the B cell surface and presentation to T cells (Figure 3A).[56] Dendritic cells that phagocytose PEG-conjugates also present the same peptides as MHC class II complexes on their surface. T_{FH} cells that recognize peptide-MHC complexes on dendritic cells proliferate and increase the expression of costimulatory molecules such as CD40L.[57] Anti-PEG B cells that migrate into secondary lymphoid follicles and interact with T_{FH} cells can form germinal centers where antibody affinity maturation and class switching occur.[58] T_{FH} cells also secrete the cytokines

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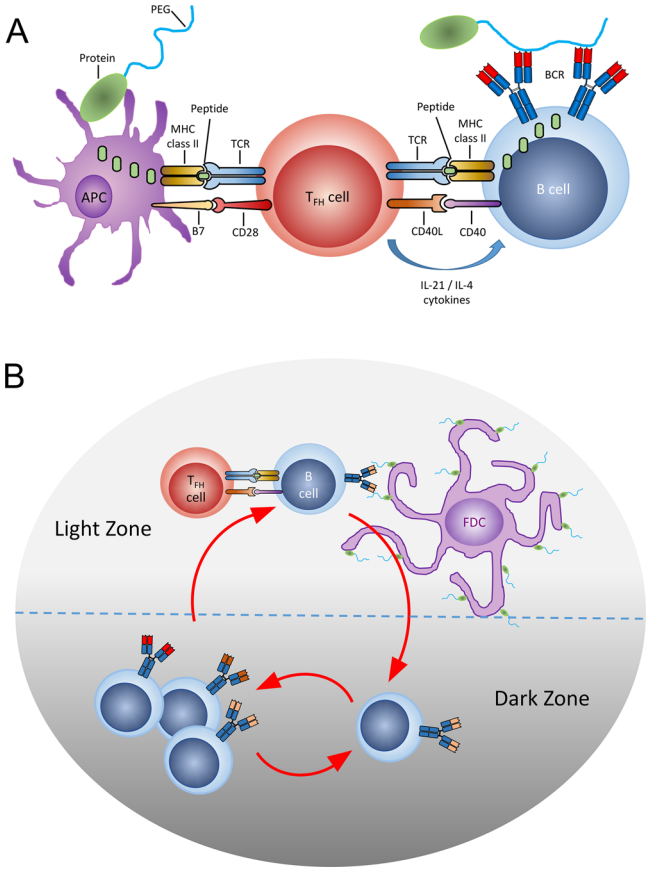

Figure 3. Thymus-dependent immune response against PEG. (A) Antigen-presenting cells take up pegylated proteins and present digested peptide fragments to activate specific T_{FH} cells. Pegylated protein that is bound by anti-PEG immunoglobulins on PEG-specific B cells is digested and presented on MHC class II molecules to receive important signals from activated T_{FH} cells (CD40L, IL-21, and other cytokines) to initiate somatic hypermutation of antibody variable region genes and class switch from IgM to IgG, IgA, or IgE in germinal centers. (B) Anti-PEG B cells undergo rapid proliferation and somatic hypermutation in the dark zone of a germinal center. B cells that display immunoglobulin with fortuitous mutations that increase PEG binding affinity can uptake greater amounts of pegylated protein from follicular dendritic cells (FDC) in the light zone. These B cells display sufficient peptide/MHC complexes to receive survival signals from T_{FH} cells and recycle back to the dark zone for additional rounds of mutation and selection in a process called affinity maturation.

interleukin-21 and interleukin-4, which promote B cell proliferation, class switch recombination, and differentiation into plasma cells or germinal center B cells.[59,60] Germinal centers are composed of a dark zone and a light zone (Figure 3B). Activated B cells in the dark zone rapidly proliferate. Interaction of CD40L on T_{FH} cells with CD40 on germinal center B cells induces the expression of activation-induced cytidine deaminase, which is required for both class-switch recombination and somatic hypermutation of antibody gene variable domains.[61,62] Error-prone repair of deaminated cytidine residues formed by the action of activation-induced cytidine deaminase results in random introduction of somatic mutations in the variable region genes of the antibodies expressed by the germinal center B cells.[56,63] Activation-induced cytidine deaminase induced lesions in the heavy-chain locus can also introduce double strand DNA breaks that result in the irreversible switch from IgM to IgG, IgA, or IgE.[64] B cells then enter the light zone where they compete for PEG on the PEGylated proteins or peptides displayed on the surface of follicular dendritic cells, a specialized mesenchymal-derived cell that is distinct from traditional dendritic cells.[65] Follicular dendritic cells express complement receptors to facilitate capture and retention of immune complexes on their surface. Anti-PEG B cells with mutations in their BCRs that result in higher affinity for PEG successfully compete with lower affinity B cells for the binding and internalization of limiting amounts of pegylated protein on follicular dendritic cells. Besides generating stronger signaling through the BCR, these B cells internalize larger quantities of conjugate and display higher levels of peptide MHC complexes on their surface to more effectively interact with and receive help from T_{FH} cells, which is required to prevent apoptosis of the B cells.[66,67] B cells that receive sufficient T_{FH} help survive, recycle back to the dark zone where they can divide and undergo additional rounds of somatic hypermutation and affinity selection.[67−69] After several rounds of somatic hypermutation in the dark zone and selection for high-affinity clones in the light zone, germinal center B cells can differentiate into long-lived memory B cells or plasma cells that lose surface expression of immunoglobulin but can secrete soluble antibodies.[69] Note that B cells that secrete antibodies against PEG recognize the PEG portion of pegylated conjugate, whereas T_{FH} cells recognize the protein portion of the conjugate (as peptide-MHC complexes).

B and T cells undergo selection processes during their development that limit reactivity against self- antigens.[70,71] Immature B cells that strongly bind to self-antigens undergo receptor editing to change their binding specificity. Those that retain binding to self-antigens undergo apoptosis. Likewise, immature T cells go through a process of thymic selection to generate mature T cells. T cells are positively selected for binding to self MHC molecules. T cells that do not bind self MHC molecules undergo apoptosis, resulting in restriction of T cell responses to cells expressing self MHC molecules. However, T cells that bind with high affinity to self MHC-peptide complexes are also eliminated, resulting in mature T cells that are not activated by self-peptides. The net result of these selection processes is that mature B and T cells in normal individuals do not recognize self-proteins and therefore are unable to mount substantial immune responses against therapeutic proteins derived from human sources. Thymus-dependent (TD) responses against PEG are therefore only generated when PEG is linked to a non-human peptide or protein.

ANTI-PEG ANTIBODY RESPONSES TO THYMUS-INDEPENDENT ANTIGENS

Non-protein antigens can also induce antibody responses including secretion of IgM, IgG, and IgA antibodies. Non-proteins are classified as thymus-independent (TI) antigens because they cannot generate peptides for presentation by MHC class II molecules on the B cells surface to interact with T_{FH} cells. Thymus-independent type 2 (TI-2) antigens are multivalent and can extensively cross-link BCRs.[72] A single PEG polymer contains many epitopes for antibody binding and can therefore act as a TI-2 antigen (Figure 4). Marginal zone B cells are primarily responsible for the development of antibody responses to TI-2 antigens.[47]

Besides the primary signal provided by cross-linked BCRs, innate immune cells can provide additional activating signals

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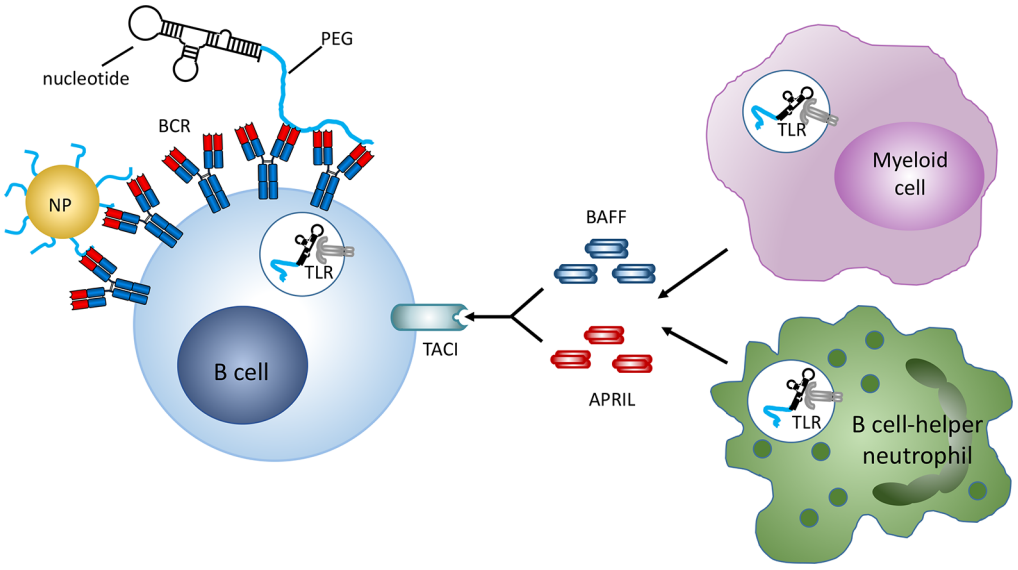

Figure 4. Thymus-independent immune response against PEG. Cross-linking of multiple anti-PEG immunoglobulins (BCRs) on a PEG-specific B cell can induce a weak TI-2 response, which can be amplified by co-activation of innate immune cells *via* interaction with toll-like receptors (TLR) to promote secretion of B cell-activating factor of the tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL), which interact with transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) on B cells.

Table 5. Predicted Immune Responses to Pegylated Compounds in Humans

		immunogenicity in humans	
pegylated compound	examples	TD response	TI response
human proteins	pegfilgrastim, peginterferon alfa-2a	–	+/–
human protein replacement	PEG-antihemophilic factor, coagulation factor IX	++	+/–
foreign proteins	pegaspargase, pegloticase	++++	+/–
nucleic acids	pegaptanib	–	+/–
empty liposome or nanoparticles	empty liposomes	–	+
cytotoxic drug liposomes	pegylated liposomal doxorubicin (PLD)	–	–
nucleic acid nanoparticles	patisiran, toxinameran, mRNA-1273	–	+++

via toll-like receptors (TLR) and transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI). The most important signals are provided by B cell-activating factor of the tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL). These members of the tumor necrosis factor family are synthesized as type II transmembrane proteins that are proteolytically cleaved intracellularly or on the cell surface to release soluble trimeric and higher order active forms.[73,74] APRIL and BAFF are released by dendritic cells and marginal zone macrophages in mice and by B-cell helper neutrophils in humans.[47,75] BAFF and APRIL levels are increased in the presence of type I interferons, interferon gamma, interleukin-10, granulocyte colony-stimulating factor, and ligands that cause signaling through TLRs.[76,77] Interaction of BAFF and APRIL with TACI is required for inducing the expression of activation-induced cytidine deaminase in marginal zone B cells for class switching against TI-2 antigens.[78−80] Mouse marginal zone B cells can therefore produce IgM, IgG_{2b}, IgG_3, and IgA, whereas human marginal zone B cells can produce IgM, IgG_1, IgG_2, and IgA_2 in response to T cell-independent antigens.[47,75] B-cell helper neutrophils may also induce somatic hypermutation of antibody genes in marginal zone B cells.[81]

The relative propensity of pegylated compounds to generate antibodies against PEG can be estimated based on the properties of the compound physically attached to PEG. Examples of several classes of compounds and the expected PEG immunogenicity are listed in Table 5. The classes of pegylated compound are discussed below.

HUMAN PROTEINS

Pegylated human proteins display immunogenicity in animal models due to the presence of B and T cells that recognize the human protein as foreign.[82] By contrast, pegylated recombinant human proteins rarely generate anti-PEG antibody responses because most self-reactive T and B cells are absent due to negative selection during their development. Heavily pegylated human proteins can potentially generate weak TI-2 antibody responses by cross-linking BCRs on anti-PEG B cells, but human proteins that do not directly activate innate immune cells will have limited anti-PEG antibody responses due to lack of help from neutrophils and myeloid cells. This is consistent with few reports of immunogenicity for most pegylated human proteins. It should be noted, however, that antibodies can be generated against proteins that differ from a *bona fide* human protein by only a single or few amino acids, as well as by differences in glycosylation and other post-translational modifications.[83,84] Manufacturing, purification, and formulation conditions can also affect the immunogenicity of recombinant human proteins.[85,86] As more generic forms of pegylated medicines come on the market, additional scrutiny of possible immunogenicity due to subtle differences in manufacturing processes may be warranted.[87]

The use of a pegylated proteins to replace a deficient protein is an important exception to the low immunogenicity of human proteins. Patients that completely lack an endogenous protein possess B and T cells that did not undergo negative selection against the missing protein. B cells can therefore receive help

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from T_{FH} cells to generate antibody responses. This phenomenon is observed in previously untreated patients suffering from severe forms of hemophilia A where neutralizing IgG antibodies against factor VIII are induced in 30−35% of individuals.[88,89] Mutations that cause large deletions or loss of factor VIII protein expression are associated with a higher incidence of antibodies (up to 88%), whereas mutations that cause loss of factor VIII function but allow some FVIII protein production are associated with a lower incidence of antibody formation (3−10%), consistent with lack of B and T cell tolerance in patients with little production of endogenous factor VIII.[90,91] Besides antibody responses to the replacement protein, anti-PEG antibodies can also be generated. Among 207 pre-treated patients suffering from severe hemophilia A, 13 patients developed anti-PEG antibodies during treatment with BAY 94-9027 (Jivi, a recombinant B-domain deleted human factor VIII produced in BHK cells and modified with a branched $PEG_{60\,000}$ molecule).[92] In a study of 270 severe hemophilia A patients receiving multiple doses of turoctocog alpha pegol (N8-GP, a recombinant B-domain-truncated human factor VIII produced in CHO cells in which a single branched $PEG_{40\,000}$ chain is attached to a glycan on factor VIII), 32 patients had pre-existing antibodies against PEG, which increased to 45 patients after initiation of N8-GP treatment. Two individuals that withdrew from the study had pre-existing or developed anti-PEG antibodies during the study.[93] These early clinical trials are limited to previously treated patients without detectable FVIII inhibitory antibodies, which means that these patients retain tolerance against FVIII. The incidence of induced anti-PEG antibodies may be greater in patients who do not express detectable levels of FVIII and have T cells that can be activated by recombinant FVIII to stimulate anti-PEG B cell responses.

FOREIGN PROTEINS

In contrast to human proteins, pegylated therapeutics derived from non-human sources can generate robust antibody responses to PEG because T_{FH} cells are present that can be activated by peptides derived from the therapeutic protein. Prominent examples include pegaspargase, pegloticase, and pegvaliase.

Pegaspargase is a recombinant form of L-asparaginase derived from *E. coli* and modified with 69−82 chains of PEG_{5000}. Pegaspargase is used to reduce the levels of serum asparagine, which is essential for the survival of some leukemia cells due to a metabolic defect in asparagine synthesis.[94] Pegaspargase was granted FDA approval in 2004 for patients with acute lymphoblastic leukemia who are hypersensitive to L-asparaginase and in 2006 for the first-line treatment of patients with acute lymphoblastic leukemia.[95] In a landmark study on the impact of anti-PEG antibodies on a pegylated drug, anti-PEG IgM or IgG antibodies were detected in 46% and 32% of patients treated with pegaspargase, respectively.[7]

Pegloticase is a recombinant porcine uricase that is modified with nine molecules of $PEG_{10\,000}$ and was approved by the FDA in 2010 for the treatment of severe, treatment-refractory, chronic gout. Pegloticase reduces urate levels by enzymatic conversion of urate to the more soluble allantoin.[96] Five of 18 gout subjects that received a single subcutaneous injections of PEG-uricase developed anti-PEG IgM antibodies within 3−7 days and anti-PEG IgG within 7 days of drug administration.[96] In a study in which the route of administration was switched from subcutaneous to a single intravenous injection of pegloticase, nine of 24 subjects developed anti-PEG IgG antibodies, mostly of the IgG_2 subclass.[50] Similarly, 40% (67/169) of patients receiving biweekly intravenous infusions of pegloticase developed anti-PEG antibodies,[97] and 13 of 30 gout patients treated intravenously with pegloticase every 3 weeks developed antibodies that bound to the repeating ethylene oxide backbone of PEG.[6]

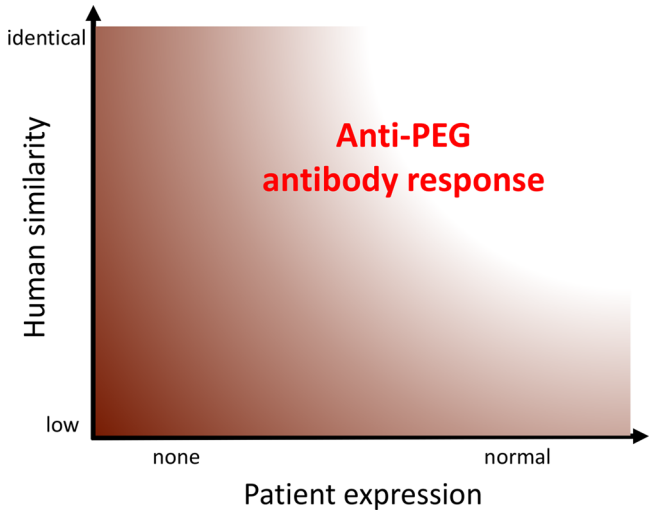

Figure 5. Anti-PEG antibody responses to TD antigens. PEG-modified recombinant proteins that are identical in every way to normal human proteins and are expressed at normal levels in patients do not generate antibodies against PEG. Totally human proteins, however, can induce anti-PEG antibodies in patients lacking the protein. Likewise, human proteins that differ from their native counterparts as well as foreign proteins can induce anti-PEG antibody responses.

Phenylketonuria is an inherited disease caused by low phenylalanine hydroxylase activity which leads to accumulation of neurotoxic levels of phenylalanine. In 2018, the FDA approved pegvaliase, a recombinant cyanobacteria phenylalanine ammonia lyase produced in *E. coli* and modified with multiple $PEG_{20\,000}$ chains, to relieve the symptoms of phenylketonuria. In a study of 25 phenylketonuria patients receiving a single subcutaneous injection of pegvaliase, four patients had pre-existing antibodies to PEG, and all patients developed anti-PEG IgG antibodies.[98] In a larger study of 261 phenylketonuria patients receiving multiple subcutaneous injections of pegvaliase, 96% of the patients developed anti-PEG IgG or IgM antibodies that peaked around weeks 8−12, but the anti-PEG titers then dropped in most patients.[99]

Pegylated peptides derived from non-human sources can also induce strong anti-peptide and anti-PEG responses.[100,101] For example, intravenous injection of mice with pegylated melittin, a peptide derived from bee venom, induces IgG and IgM antibodies against both the peptide and PEG because the peptide can activate T_{FH} cells to promote affinity maturation and class switch of anti-PEG antibodies in germinal centers.[101] Interestingly, replacement of naturally occurring L-amino acids with the corresponding D-amino acid largely prevents the formation of antibody responses to both melittin and PEG,[101] likely due to poor presentation of D-amino acid peptides by MHC class II molecules for activation of T_{FH} cells.[102] This approach may be generally useful for peptides that can tolerate substitution with D-amino acids and also suggests that other

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approaches to reduce peptide and protein immunogenicity may reduce anti-PEG responses.[103,104]

The induction of anti-PEG antibodies by TD antigens is summarized in Figure 5. PEG-modified recombinant proteins that are identical to normal human proteins and are expressed at normal levels in patients do not typically generate antibodies against PEG. Totally human proteins, however, can induce progressively stronger anti-PEG antibodies in patients who express truncated protein or totally lack the endogenous protein. Recombinant human proteins that differ in sequence, post-translational modifications, or aggregation status can induce anti-PEG antibody responses as they become more dissimilar to their native human counterpart. Non-human foreign proteins typically induce strong anti-PEG antibody responses. Individual differences in each component of the immune system, especially expression of specific major histocompatibility complex alleles, can influence how immunogenic a particular protein appears to an individual patient.[83,105,106]

LIPOSOMES AND NANOPARTICLES

Pegylated liposomes and nanoparticles can cross-link BCRs on PEG-specific B cells to induce production of anti-PEG IgM antibodies *via* a TI-2 response.[107,108] The thymus-independent nature of the response is shown by induction of anti-PEG antibodies in BALB/c nude mice which lack T cells.[109] Marginal zone B cells are responsible for generating anti-PEG IgM antibodies against pegylated nanoparticles in mice.[110,111] Anti-PEG IgM induced by the first injection of pegylated liposomes can activate complement, facilitating binding of later administrations of liposomes by complement receptors present on marginal zone B cells.[112] Induction of anti-PEG antibodies follows a bell-shaped dose–response, with strong antibody production at intermediate but not at low and high doses of liposomes,[113−115] as found for other TI antigens.[116,117] Pre-administration of empty liposomes (Doxebo) has been proposed to reduce infusion reactions to pegylated drugs, but it remains to be confirmed that sufficient doses can be infused to prevent generation of anti-PEG antibody responses.[118,119]

In contrast to empty liposomes, administration of clinically relevant doses of pegylated liposomes that encapsulate cytotoxic drugs do not induce anti-PEG antibody responses. Pegylated liposomal doxorubicin (PLD) prevents the generation of high levels of anti-PEG IgM and prolongs the half-life of a second dose of PLD.[114,120] Lack of anti-PEG IgM responses was verified in beagle dogs that received clinically relevant doses of PLD.[121] Injection of therapeutic doses of pegylated liposomes loaded with the anticancer drugs mitoxantrone or oxaliplatin also do not induce anti-PEG IgM antibody responses.[122−124] On the other hand, anti-PEG antibodies responses occur in some animal models when low doses of liposome are administered or when liposomes encapsulate specific drugs.[121,124−127]

Attachment of cytotoxic drugs to dextran, a TI-2 antigen, selectively depletes anti-dextran producing B cells and blocks induction of antibodies against subsequently administered dextran.[128] Antibody responses against proteins attached to the surface of liposomes are also blocked by pre-administration of the same liposome containing encapsulated doxorubicin.[129,130] These studies suggest that the absence of anti-PEG antibody responses against clinically relevant doses of cytotoxic pegylated liposomes is caused by binding and endocytosis of

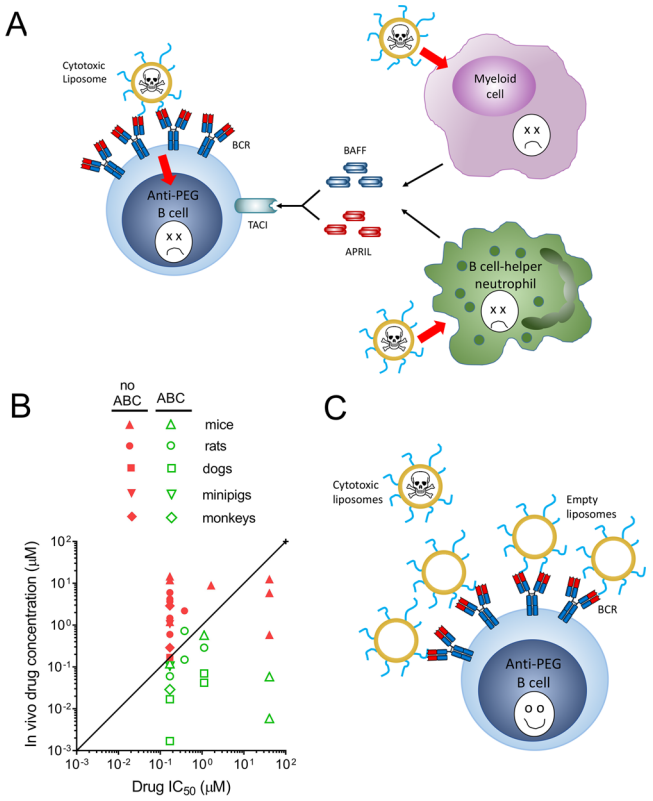

Figure 6. Relation between drug potency and induction of accelerated blood clearance by cytotoxic pegylated liposomes. (A) Pegylated liposomes that encapsulate cytotoxic payloads bind anti-PEG immunoglobulins on marginal zone B cells, resulting in selective killing of PEG-specific B cells. Phagocytosis of the liposomes by innate immune cells may also blunt anti-PEG antibody responses. (B) Graph showing the estimated total *in vivo* drug concentration provided by pegylated liposomes *versus* drug potency as described in Supplemental Table 2. Experimentally observed ABC is shown by open green symbols, whereas lack of ABC is shown in filled red symbols.[114,121−127,131] ABC (reflecting anti-PEG antibodies) is not observed when drug dose exceeds drug potency. (C) Empty pegylated liposomes can compete with cytotoxic liposomes for binding to PEG specific B cells, preventing uptake of sufficient cytotoxic liposomes to deplete anti-PEG B cells.

the liposomes by PEG-specific marginal zone B cells, resulting in selective depletion of anti-PEG B cells (Figure 6A).[15,122] Phagocytosis of cytotoxic liposomes may also deplete innate immune cells involved in the TI-2 humoral immune response. Low doses of cytotoxic liposomes cannot achieve sufficient concentrations to kill marginal zone B cells. Indeed, accelerated blood clearance (which is a surrogate marker of anti-PEG antibody levels as discussed below) does not occur when liposomes encapsulate cytotoxic levels of drugs, which we estimate by plotting *in vivo* drug concentration (calculated as total drug dose divided by the blood volume) *versus* the potency of the encapsulated drug (estimated as IC_{50} values against cancer cells) (Supplemental Table 2) (Figure 6B). Even though this rough estimate does not consider drug uptake by specific anti-PEG marginal zone B cells or species differences in drug sensitivity, the prediction holds well against all species of animals tested. Cytotoxic drug needs to be encapsulated in the liposomes to effectively suppress anti-PEG antibody responses as shown for topotecan liposomes, which

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rapidly release drug *in vivo*; it is estimated that shortly after administration only about 6% of the liposomes retain topotecan, resulting in accelerated clearance at drug concentrations that are expected to kill B cells if the drug is retained inside liposomes.[127,131] Co-injection of excess empty liposomes increases the induction of anti-PEG antibodies, consistent with competitive blocking of cytotoxic liposome uptake into marginal zone B cells (Figure 6C).[121]

NUCLEIC ACIDS

Pegylated nucleic acids cannot mount a TD response but can induce TI-2 responses since nucleic acids can directly activate innate immune cells. Short RNA molecules can activate TLR3, TLR7, and TLR8 and the RNA helicase retinoic acid-inducible gene I to elicit the secretion of interferon alpha and other inflammatory cytokines.[132] Likewise, CpG motifs in DNA and RNA molecules such as aptamers can activate TLRs.[100,133] However, the immunogenicity of nucleotide drugs can be reduced by selecting functional sequences that display low immunostimulatory capacity and by introducing chemical modifications such as 2′-fluoro-modified pyrimidines and 2′-*O*-methyl-modified purines.[134−137]

Pegaptanib is a 27 base aptamer that contains a phosphorothioate 3′-3′ deoxythymidine cap to hinder nuclease degradation, 2′-*O*-methylation modification of the purine ribose sugars and 2′-fluorination of the pyrimidine ribose sugars.[138,139] A branched $PEG_{40\,000}$ molecule is attached to the 5′ end of the aptamer, which is approved by the FDA to treat age-related macular degeneration (AMD) of the retina. There are no reports of anti-PEG responses, but pegaptanib is directly injected into the eye, which is considered to be an immune-privileged site.[140] ARC1779 is an aptamer conjugated to $PEG_{20\,000}$ which blocks platelet activation by inhibition of von Willebrand factor binding to the glycoprotein Ib-IX-V receptor complex. It is unclear if a case of hypersensitivity to ARC1779 is related to anti-PEG antibodies.[141] Clinical trials of ARC19499, an aptamer linked to $PEG_{40\,000}$ which blocks tissue factor pathway inhibitor (TFPI), were terminated due increased bleeding events in hemophilia patients at high drug doses, but this was apparently unrelated to anti-PEG antibody responses.[142,143] No immune related events were observed in a phase IIa clinical trial of olaptesed pegol, a pegylated L-oligoribonucleotide which binds and neutralizes the chemokine CXCL12 for the treatment of chronic lymphocytic leukemia.[144] Likewise, no treatment-related serious adverse events occurred in early clinical trials of emapticap pegol, a 40 L-nucleotide aptamer that binds human monocyte chemoattractant protein.[145] Taken together, these studies indicate that pegylated nucleic acid drugs can be cleverly designed to minimize anti-PEG antibody responses.

LIPID NANOPARTICLES AND SARS-CoV-2 VACCINES

Nanoparticles that encapsulate DNA or RNA can generate strong anti-PEG antibody responses. PEG-coated lipoplexes containing plasmid DNA with CpG motifs induced strong cytokine production and anti-PEG IgM responses in mice.[146] Anti-PEG antibody responses were greatly reduced in mice lacking MyD88 (an adaptor protein that links TLR signaling to downstream effector functions) or TLR9 (which recognizes unmethylated DNA with CpG motifs), consistent with a TI-2 antibody response.[146] Pegylated liposomes encapsulating oligonucleotides generate greater anti-PEG antibody responses

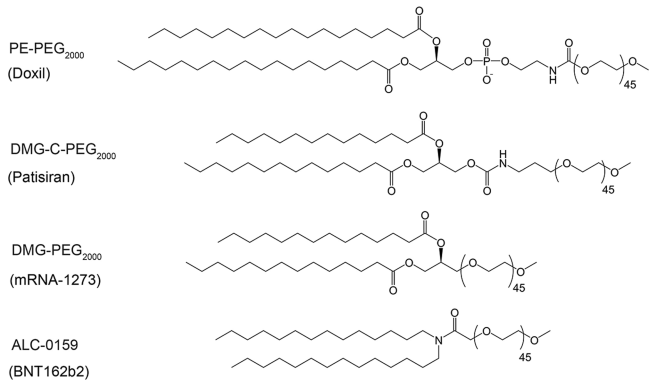

Figure 7. PEG lipids used in liposomes and lipid nanoparticles. PE-PEG_{2000} possesses a charged C18 lipid tail and stably associates in lipid particles. The other lipids possess neutral C14 lipid tails that spontaneously release from lipid nanoparticles at physiological conditions.

in mice as compared to empty pegylated liposomes, even for nucleotides that do not have immunostimulatory CpG motifs.[109] Encapsulation of DNA and RNA molecules may facilitate endocytosis and produce differential immunogenicity as compared to free oligonucleotides.

Generation of anti-PEG antibodies against lipid nanoparticles can be reduced by using "sheddable" PEG on the lipid surface. Stealth liposomes such as pegylated liposomal doxorubicin or irinotecan liposomes incorporate 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine−poly(ethylene glycol 2000) (PE-PEG_{2000}) that is stably anchored in the lipid bilayer (Figure 7).[147,148] By contrast, PEG lipids that have shorter tails remain in the lipid particles during manufacture and storage but are rapidly released *in vivo via* exchange with lipoprotein particles in plasma.[149,150] For example, PE-PEG_{2000} remains associated in lipids with a half-life of ∼25 h, whereas a neutral-lipid PEG molecule with a 14-carbon tail (DMG-PEG_{2000}) is rapidly released with a half-life of ∼1.3 h.[151] Besides allowing greater interaction of the lipid nanoparticle with target cells,[152,153] loss of PEG from the particles reduces cross-linking of immunoglobulin on the surface of PEG-specific B cells to limit induction of TI-2 immune responses.

Patisiran, used to treat polyneuropathy caused by transthyretin amyloidosis, is a 21-mer double-stranded small interfering RNA molecule containing 2′-*O*-methyl-modified and unmodified ribonucleosides, with 2′-deoxythymidine dinucleotide overhangs at the 3′ ends that is encapsulated in cationic lipid nanoparticles coated with 1,2-dimyristoyl-*rac*-glycero-3-carbonylaminoethyl-*ω*-methoxypolyethylene glycol-2000 (DMG-C-PEG_{2000}, Figure 7).[154] A total of 3.4% of patients receiving patisiran developed antibodies against PEG, but the response was transient, becoming negative between 18 weeks and 18 months after initiation of treatment.[155]

Two SARS-CoV-2 vaccines currently approved for emergency medical use (BNT162b developed by BioNTech, Pfizer, and Fosun Pharmaceutical and mRNA-1273 from Moderna) contain nucleoside-modified messenger RNA (mRNA) in lipid nanoparticles with PEG_{2000} attached to their surface *via* neutral lipids with lipid tails containing 14 carbons (Figure 7).[3,4] BNT162b and mRNA-1273 are safe in the vast majority of recipients.[156] However, with the widespread rollout of the vaccines, there have been concerns that pre-existing anti-PEG antibodies may cause allergic reactions (anaphylactoid reactions) in some individuals after receiving the first dose of

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vaccine.[157] However, of potentially greater concern is possible widespread development of anti-PEG antibodies in vaccinated individuals, which may affect the efficacy and safety of other pegylated medicines. No data is currently available on whether these vaccines induce antibodies against PEG, but if 3.4% of the population generate anti-PEG antibody responses (similar to the incidence in patients receiving patisiran), this may be a game changer for pegylated therapies. There are many differences between patisiran and the vaccines. The dose of patisiran (up to 30 mg) is much greater than the doses of mRNA-1273 (100 μg) and BNT162b2 (30 μg). Patisiran is administered intravenously every 3 weeks, whereas mRNA-1273 and BNT162b2 are given as two intramuscular injections with a month-long interlude. On the other hand, the vaccines are designed to induce immune responses. It is important that the frequency, strength, and duration of possible anti-PEG antibody responses to mRNA-1273 and BNT162b2 are determined as soon as possible, since induced anti-PEG antibodies may alter how other pegylated medicines act in patients.

ACCELERATED BLOOD CLEARANCE

Accelerated blood clearance (ABC) of pegylated compounds has been extensively documented in both animal models and patients. Anti-PEG antibodies were found in 1999 to induce ABC of pegylated proteins in mice and in humans in 2007 as an association between induction of anti-PEG antibodies with loss of asparaginase activity in patients treated with pegaspargase.[7,17,158] Rapid drug clearance and loss of drug efficacy also occur in patients who develop anti-PEG IgM and IgG antibodies after receiving PEG-uricase[6,50,96,97] and in phenylketonuria patients receiving multiple subcutaneous injections of pegvaliase.[99] ABC of pegylated liposomes was reported in 2000,[159] with many subsequent studies in animal models confirming that administration of empty pegylated liposomes can induce anti-PEG antibodies that cause ABC of subsequently administered pegylated liposomes.[107−109] Other nanoparticles including PEG-modified PLA nanoparticles, pegylated microbubbles, and PEG-coated lipoplexes also induce anti-PEG antibody responses and ABC in animal models.[160,161] Mice models mimicking the presence of pre-existing anti-PEG antibodies at physiological relevant antibody concentrations show that both anti-PEG IgM and IgG antibodies produce strong ABC of pegylated liposomal doxorubicin, resulting in reduced tumor localization and loss of anti-tumor activity.[23]

Clearance of pegylated compounds is caused by formation of immune complexes between anti-PEG antibodies and the pegylated compound, resulting in complement activation, deposition of complement reaction products on the immune complex, and phagocytosis into resident macrophages (Kupffer cells) in the liver (Figure 8).[107,146,159] Anti-PEG antibodies can also hinder the distribution of pegylated nanoparticles to target tissues. For example, *N*-linked glycans present on anti-PEG antibodies bound to pegylated nanoparticles interact with mucin in the mucosal layer and prevent passage to the epithelial surfaces.[162]

Some pegylated nanomaterials and proteins do not display ABC in animal models. For example, minimal ABC is observed for micelles after priming with PEG liposomes even though the micelles induce anti-PEG antibodies and strong ABC of PEG liposome in rats.[163−165] Likewise, pegylated PLGA nanoparticles induce anti-PEG IgM antibodies and ABC for a second dose of PEG-PLGA nanoparticles but produce only moderate ABC for pegylated liposomes and no ABC for BSA that was modified with 17−20 PEG_{5000} chains.[166]

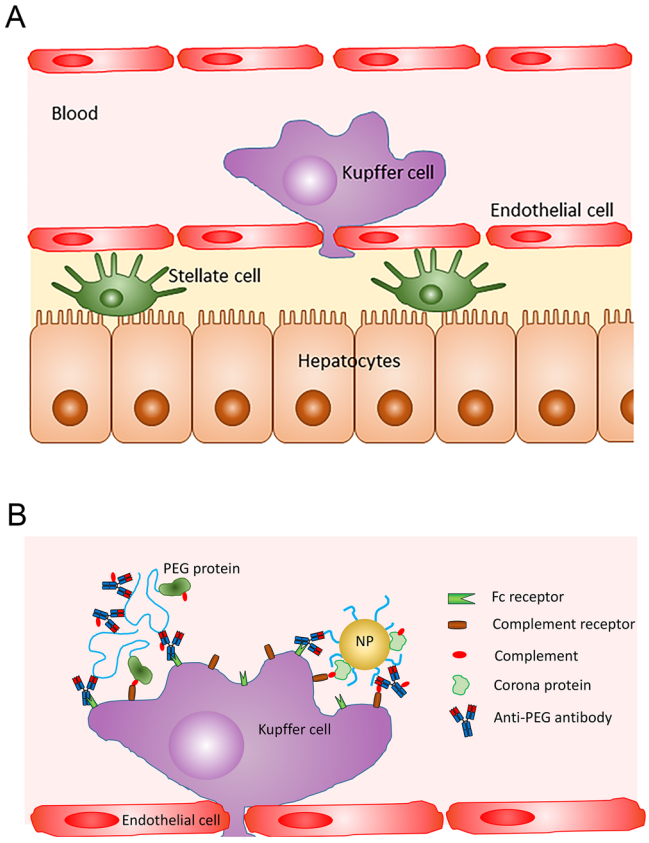

Figure 8. Clearance of immune complexes in the liver. (A) Illustration of resident macrophages (Kupffer cells) in the liver. (B) Immune complexes formed between pegylated compounds and anti-PEG antibodies can activate the complement system, resulting in deposition of complement components on proteins, including anti-PEG antibodies and proteins adsorbed to the surface of nanoparticles. Kupffer cells initiate phagocytosis of immune complexes and opsonized nanoparticles *via* Fc receptor binding of anti-PEG antibodies and complement receptor binding of complement. Drawing is not to scale.

Several mechanisms have been proposed to explain why some pegylated nanomedicines appear to be immune from ABC, including different architectures of PEG on NPs *versus* proteins,[166] inability of nanoparticles below a critical size to be cleared by anti-PEG antibodies,[164] induction of anti-PEG IgM antibodies with different specificities and epitope affinities to PEG,[100] or the requirement of an interface between hydrophilic PEG chain and hydrophobic blocks on PEG-conjugates for anti-PEG antibody binding.[167] A simpler explanation is that effective ABC requires a threshold molar ratio of anti-PEG antibodies to PEG compound (Figure 9A).[168,169] The number of proteins or micelles greatly exceeds the number of liposomes injected for the same administered dose on a mass basis. For example, 5 mg kg^{-1} of 96 nm liposomes corresponds to 1×10^{13} liposomes/rat, whereas 5 mg kg^{-1} of 30 nm PEG−DSPE:PC micelles corresponds to 1.7×10^{15} micelles/rat.[166] At a typical anti-PEG IgM concentration of 10 μg mL^{-1}, this corresponds to 7.7 IgM molecules per liposome but only 0.045 IgM per micelle. Thus, most micelles are not bound by IgM and are not cleared from the circulation. Analysis of previous

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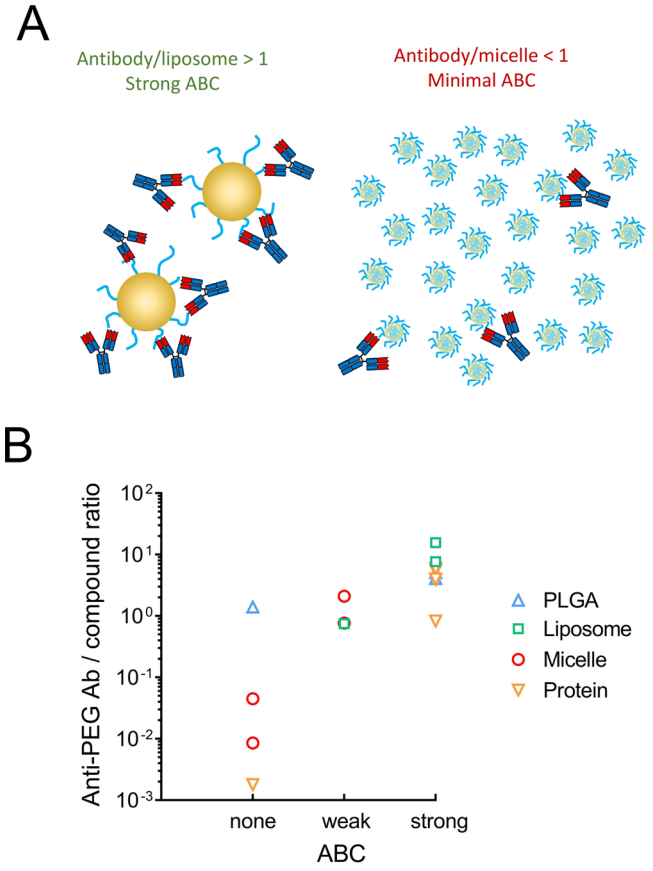

Figure 9. Ratio of anti-PEG antibodies to injected drug dose determines ABC. (A) Illustration of an example where ABC is observed for liposomes (left panel), whereas the same mass of micelles does not induce ABC (right panel). (B) The estimated number of anti-PEG antibodies per pegylated compound *in vivo* is estimated and shown for cases in which ABC was absent (none), moderate (weak), or robust (strong).[17,164,166,169] Details are listed in Supplemental Table 3.

studies (Supplemental Table 3) shows that strong ABC is observed when the number of antibodies in circulation exceeds the number of pegylated compound (Figure 9B). This holds for pegylated proteins, liposomes, micelles, and polymeric nanoparticles and agrees with previous studies showing that three anti-PEG antibodies per pegylated protein or about 10 anti-PEG antibodies per pegylated liposome are required for ABC.[25,168,169] Administration of large numbers of smaller nanoparticles or micelles may be advantageous to "dose through" moderate concentrations of anti-PEG antibodies.

The incidence of pre-existing anti-PEG antibodies is high in the general population, but the concentrations of anti-PEG antibodies are very low in most positive individuals (Figure 2). Pre-existing antibodies against PEG are therefore more likely to cause ABC of pegylated drugs administered at low molar doses. Based on our study of 2400 normal donors in Taiwan, only rare individuals have concentrations of anti-PEG IgM (0.04% of the general population) that exceed 25 μg mL^{-1} (Figure 10A) or of anti-PEG IgG (0.17% of the population) that exceed 100 μg mL^{-1} (Figure 10B). On the other hand, about 1% of the population have anti-PEG IgM, and over 6% have IgG at levels exceeding 5 μg mL^{-1}. These antibody concentrations may be clinically important since peak serum concentrations of some pegylated medicines such as cytokines and interleukins are in the upper pg mL^{-1} to low μg mL^{-1} range.[170−172] A rough estimate of the percentage of the general population that might be susceptible to ABC for different pegylated medicines is shown in Figure 10C for anti-PEG IgM and Figure 10D for anti-PEG IgG. This analysis suggests that pre-existing anti-PEG IgM is not important for ABC and that pegylated interferons and epoetin-beta are most susceptible to ABC induced by anti-PEG IgG antibodies. This is consistent with reports that pre-existing anti-PEG antibodies decrease the anti-viral activity of PEG-interferon-α by accelerating clearance from the blood *via* uptake into Kupffer cells in the liver and an increased incidence of anti-PEG antibodies in patients who do not respond to PEG-epoetin-beta for the treatment of anemia associated with chronic kidney disease.[25,173]

Several factors complicate attempts to extrapolate data obtained in animal models to the clinic. Most clearance studies are performed in rodent models, which differ from humans in their complement activity, mechanisms of immune complex clearance, and ability of various antibody subclasses to interact with Fc receptors on phagocytes.[174−176] In addition, anti-PEG antibody concentrations in patient samples are calculated by comparison against a known concentration of standard antibody (Figure 11A). Since anti-PEG antibody assays tend to use high-affinity antibody standards,[21,41] the concentrations of low-affinity anti-PEG antibodies in samples are underestimated. The amount of anti-PEG antibody required to induce ABC also depends on antibody affinity (Figure 11B). High-affinity antibodies induce ABC when the molar ratio of anti-PEG antibody exceeds pegylated compound by about 3−10.[25,168,169] However, a higher molar ratio is required for low-affinity antibodies: up to 15 low-affinity IgM antibodies and over 90 low-affinity IgG antibodies are required to induce strong ABC of PEG-epoetin-beta in mice.[177] These counteracting factors make estimation of the effects of pre-existing anti-PEG antibodies especially difficult since they possess relatively low affinity for PEG. This is an area of active research that may become more important as increased numbers of people receive pegylated RNA vaccines.

DRUG RELEASE FROM LIPOSOMES

Binding of anti-PEG antibodies to PEG on the surface of liposomes can activate complement and destabilize liposome integrity. Rabbits pre-injected with pegylated liposomes to induce anti-PEG IgG antibodies cause release of fluorescence from subsequently administered pegylated liposomes containing carboxyfluorescein.[178] Anti-PEG IgM antibodies induced by pre-treatment of rats with pegylated liposomes also activate the complement cascade and accelerate release of epirubicin from pegylated liposomes.[126] Human, mouse, and rat anti-PEG IgG and IgM antibodies activate the complement cascade in the presence of PLD, resulting in destabilization of liposomal membranes and rapid leakage of encapsulated doxorubicin *in vitro* and *in vivo* (Figure 12).[24]

Complement is activated by three related pathways. Binding of an antibody to an antigen causes conformational changes in antibody structure that allow the CH_2 regions of IgG and IgM to bind C1q, the hexameric molecule responsible for the first step of the classical pathway.[179,180] The classical pathway requires physical binding of a single IgM pentamer or at least two (and optimally six) adjacent IgG molecules for effective C1q binding and activation.[181,182] The lectin pathway is analogous to the classical pathway, but instead of antibodies, carbohydrates on pathogens are bound by mannose binding

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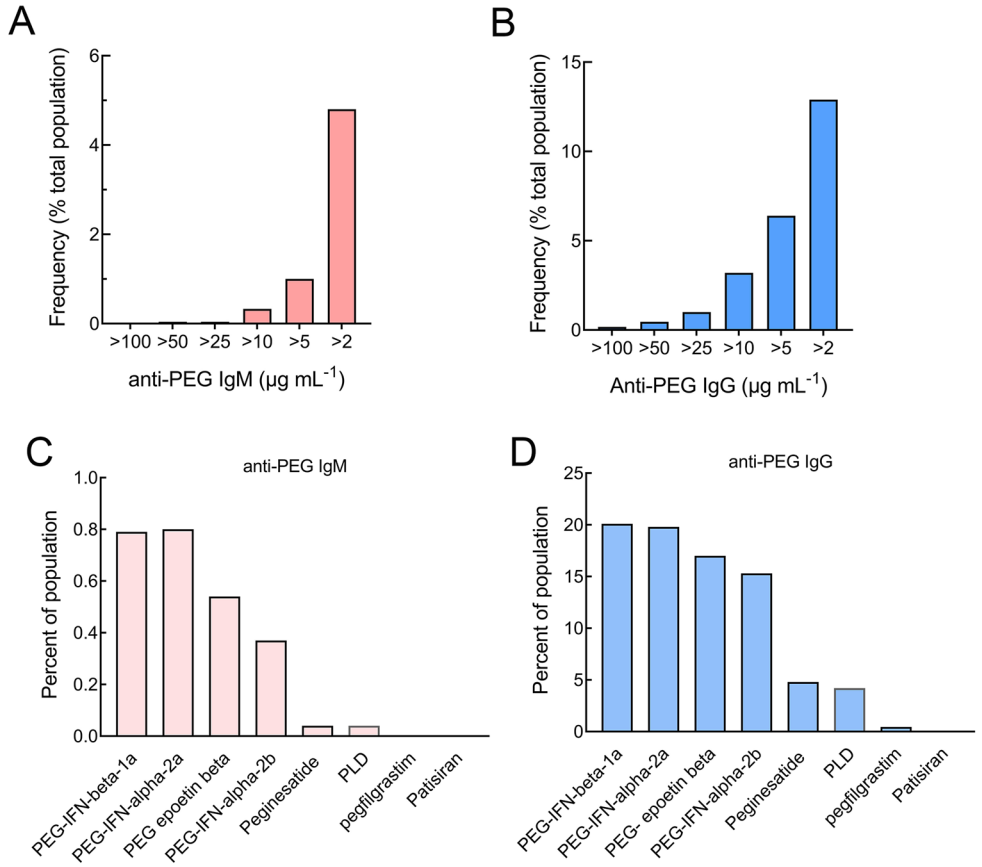

Figure 10. Concentrations and possible effective ranges of pre-existing anti-PEG antibodies. The estimated percentage of the general population with pre-existing anti-PEG IgM (A) or anti-PEG IgG (B) antibodies above the indicated concentrations. Estimate of the percentage of naive individuals with sufficient concentrations of anti-PEG IgM (C) or anti-PEG IgG (D) antibodies to experience ABC of the indicated pegylated medicines. Estimates are calculated as described in Supplemental Table 4.

lectin or ficolins.[183] The alternative pathway of complement activation spontaneously occurs at low levels but is accelerated through binding of the complement protein C3 to surfaces on biomaterials and nanoparticles.[184] This pathway plays a major role in amplifying complement activation initiated by the classical and lectin pathways.[183] The end product of all complement pathways is the formation of a membrane attack complex (C5b-9), a barrel-like protein complex which 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.[185]

PEG can directly activate the lectin and alternative complement pathways,[186] but anti-PEG antibodies bound to PEG on liposomes or nanoparticles more strongly activates complement.[15,112,187,188] Neun and colleagues found that some but not all anti-PEG monoclonal antibodies activate mouse complement in the presence of PLD.[189] By contrast, all mouse, rat, and human anti-PEG IgM and IgG monoclonal antibodies tested activate rat or human complement in the presence of PLD, with the exception of human IgG_4.[24] Complement activation is more efficient for high-affinity anti-PEG antibodies. Anti-PEG IgG activates complement on PLD mainly through the alternative pathway, while IgM-mediated drug lysis appears to proceed *via* both the classical and alternative pathways.[24] The concentration of anti-PEG antibodies necessary to induce drug release from PLD *in vivo* is relatively high (around 100 μg mL^{-1} IgG), suggesting that liposome destabilization should be uncommon in the general population but could be a concern in patients that have high levels of induced anti-PEG antibodies.

Cryogenic electron microscopy clearly shows the presence of large C5b-9 channels (~5−11 nm in diameter) in liposomes incubated with anti-PEG antibodies in the presence of complement (Figure 12).[24] Doxorubicin is remotely loaded into liposomes by creation of a transmembrane ammonium sulfate gradient that causes precipitation of doxorubicin as a sulfate salt in the form of a single nanorod crystal that is approximately 70 nm long and 20 nm wide.[190,191] The presence of C5b-9 pores in the liposome membrane disrupts the pH and ammonium gradients, causing rapid dissolution of doxorubicin sulfate crystals which can rapidly diffuse through the lipid bilayer of the liposome.[190] Onivyde, a liposomal formulation of irinotecan that is approved for the treatment of pancreatic cancer, has a much lower density of PEG on its surface (0.3 mol% compared to 5.3 mol% on PLD).[192,193] Whether a lower density of PEG on the surface of liposomes can prevent liposomal destabilization by anti-PEG antibodies is currently unknown.

HYPERSENSITIVITY REACTIONS

Hypersensitivity reactions including anaphylaxis after infusion of pegylated medicines are well documented in both animal and clinical studies. Anaphylaxis is a severe, life-threatening hypersensitivity reaction that occurs within minutes to hours after exposure to an allergen. The symptoms include flushing, shortness of breath, facial swelling, headaches, back pain,

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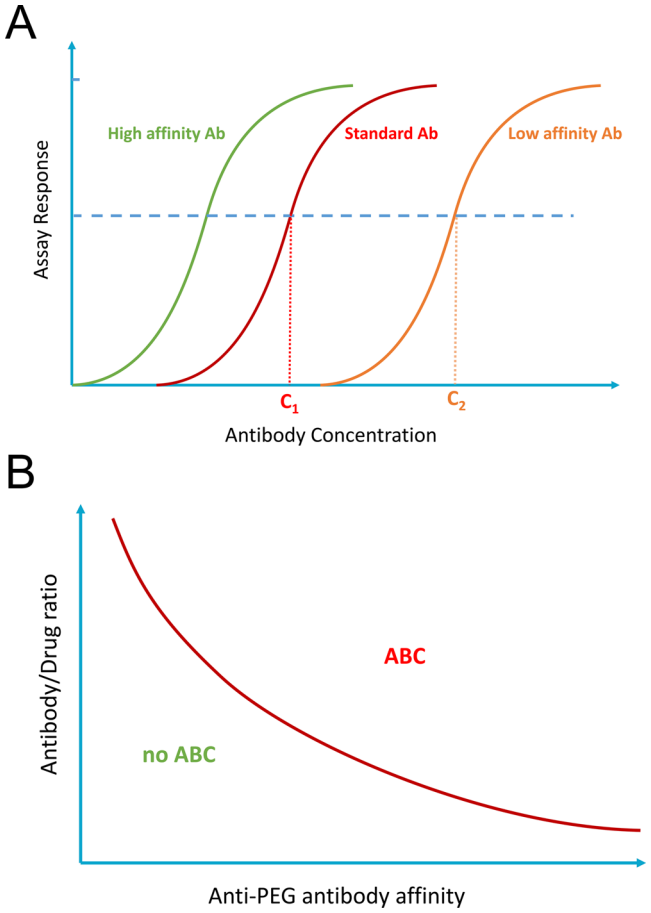

Figure 11. Effects of antibody affinity on assay estimates and accelerated blood clearance. (A) The actual concentration (C2) of a low-affinity antibody (orange curve) will be underestimated to be C1 by a higher affinity standard antibody (red curve) since only equal assay responses are compared. On the other hand, the concentration of a high-affinity antibody (green curve) will be overestimated. (B) The molar ratio of anti-PEG antibody required to cause ABC of a pegylated drug increases as the affinity of the anti-PEG antibody decreases. The antibody/drug ratio is about 3 for high-affinity antibodies.

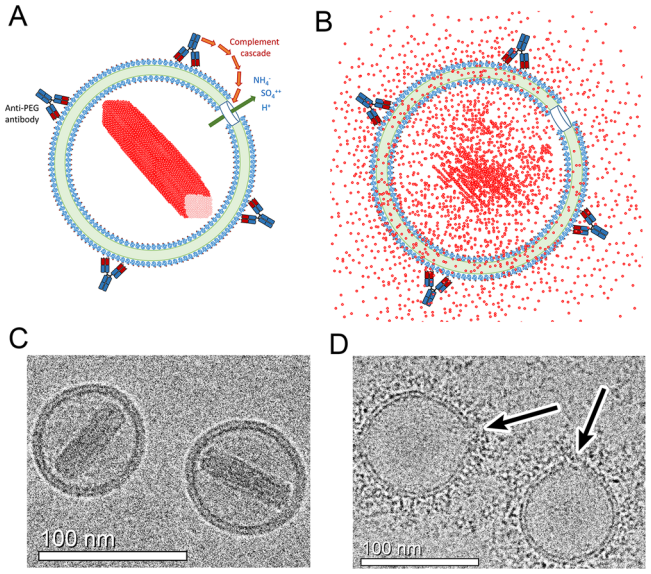

Figure 12. Anti-PEG antibodies can destabilize pegylated liposomal doxorubicin. (A) Anti-PEG antibodies that bind to PLD can activate complement and cause formation of a membrane attack complex (which forms a pore) in the liposomal membrane, breaking the internal salt and proton gradients. (B) Loss of the ammonium sulfate and proton gradients results in rapid dissolution of the doxorubicin nanocrystal and diffusion of drug from the liposomes. (C) Cryogenic electron microscopy image of PLD showing a single doxorubicin nanocrystal in each liposome. (D) Image of empty liposomes after incubation of PLD with anti-PEG IgG and complement. Arrows indicate the membrane attack complex.

tightness in the chest or throat, hypothermia and hypotension, and even death.[194]

Pegylated liposomes encapsulating oligonucleotides induce anti-PEG IgM antibodies in mice and cause anaphylactic shock upon a second injection of liposomes.[109] Likewise, pegylated liposomes that encapsulate plasmid DNA generate strong anti-PEG IgM and IgG antibody responses in mice, leading to hypersensitivity reactions including lethargy, facial puffing, vasodilation, labored respiration, and mortality.[195] Although the therapeutic benefits of PEGylated liposomal doxorubicin (PLD) is established, about 5%−10% of patients treated with PLD experience acute infusion-related hypersensitivity reactions that are potentially lethal.[196,197] A definitive link between anti-PEG antibodies and hypersensitivity reactions to PLD in patients has not been established.

Pegylated proteins that generate anti-PEG antibody responses induce hypersensitivity reactions in some patients. For example, four patients with severe hemophilia A treated with Jivi developed anti-PEG antibodies and experienced hypersensitivity reactions.[92] Infusion reactions to pegloticase are also linked to induced antibodies against PEG.[96] Thirteen of 30 gout patients treated intravenously with pegloticase every 3 weeks developed antibodies against PEG and displayed a 2-fold elevated risk of infusion reactions.[6] In a larger study, 40% of 169 patients receiving biweekly intravenous infusions of pegloticase developed anti-PEG antibodies, which was associated with the occurrence of infusion reactions.[97] Concerns regarding the safety of pegloticase led to its withdraw from the European market in 2016.

All phenylketonuria patients (25/25) receiving a single subcutaneous injection of pegvaliase developed anti-PEG IgG antibodies. Two of the subjects with the highest anti-PEG titers developed anaphylactic and hypersensitivity reactions to a PEGylated contraceptive, indicating cross-reaction of the induced antibodies to other pegylated medicines.[98] In a study of 261 phenylketonuria patients receiving multiple subcutaneous injections of pegvaliase, 96% of the patients developed anti-PEG IgG or IgM antibodies, and nearly all patients experienced hypersensitivity reactions during peak antibody levels.[99] Eleven percent of patients discontinued treatment due to adverse reactions including hypersensitivity reactions (6% of patients) with some experiencing anaphylaxis (3% of patients) or angioedema (1% of patients), arthralgia (4% of patients), generalized skin reactions lasting at least 14 days (2% of patients), and injection site reactions (1% of patients).[198]

Hypersensitivity reactions occur in 8.7−23.5% of children treated with pegaspargase, likely due to the induction of anti-PEG antibodies.[199,200] A total of 13.5% of 598 patients receiving pegaspargase developed at least one grade 2−4 reaction, and 81.5% of these patients had at least one sample that was positive for antibodies against pegaspargase with 96% of the antibodies specific to PEG.[8] Anti-PEG antibodies can

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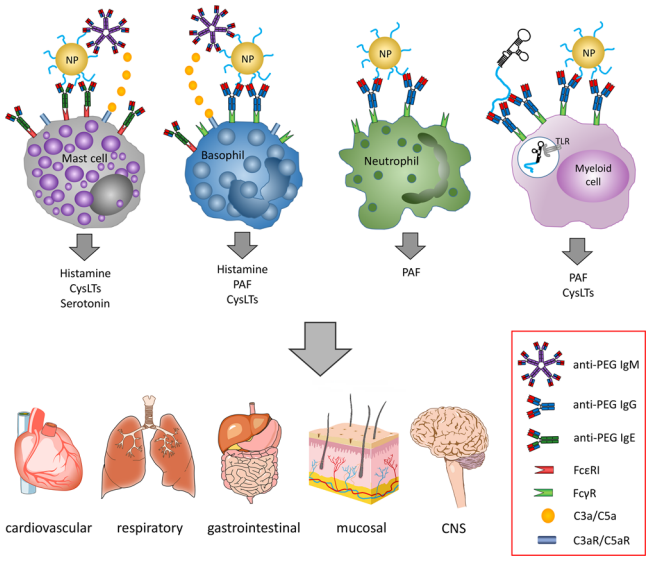

Figure 13. Possible mechanisms of hypersensitivity reactions to pegylated medicines. Anti-PEG IgG and IgM can activate the complement cascade to generate the small-peptide anaphylatoxins C3a and C5a, which can bind to C3a or C5a receptors and activate mast cells and basophils. Cross-linking of anti-PEG IgE bound to high-affinity FcεRI may also activate mast cells and basophils. Anti-PEG IgG bound to pegylated medicines may bind to Fcγ receptors and activate innate immune cells such as basophils, neutrophils, and myeloid cells. Activated cells secrete soluble mediators such as histamine, serotonin, platelet-activating factor (PAF), cysteinyl leukotrienes (CysLTs), and cytokines that can affect the cardiovascular, respiratory, gastrointestinal, mucosal, and central nervous systems. These mechanisms and pathways of hypersensitivity have not been proven for pegylated medicines.

also cross react to pegylated forms of asparaginase derived from different sources. Three patients who developed anti-PEG IgG antibodies and were allergic to pegaspargase experienced hypersensitivity reactions when treated with pegcrisantaspase, which is a pegylated form of asparaginase derived from *Erwinia*.[201] None of the patients had detectable anti-*Erwinia* asparaginase antibodies, and two patients received subsequent native *Erwinia* asparaginase without showing any evidence of clinical hypersensitivity.[8] One patient who did not develop anti-PEG antibodies tolerated three doses of pegcrisantaspase without any clinical complications.

Pre-existing antibodies against PEG can cause allergic responses to some pegylated medicines. For example, patients with pre-existing anti-PEG antibodies before treatment are at significantly higher risk of reactions to pegaspargase.[8] Pre-existing antibodies to PEG also caused acute allergic events in some patients suffering from acute coronary syndromes who were treated with the REG1 anticoagulation system, which consists of administration of pegnivacogin, a 31-nucleotide RNA aptamer conjugated to a branched $PEG_{40\,000}$ molecule designed to inhibit coagulation factor IXa, and anivacon, a complementary sequence oligonucleotide that can block the activity of pegnivacogin. A phase 2 trial of REG1 was suspended after treatment of 41 patients due to allergic-like reactions in three patients immediately after administration of pegnivacogin.[202] The three patients who experienced allergic events had elevated levels of anti-PEG IgG antibodies.[52] A phase III trial of REG1 for reduction of ischemic events during percutaneous coronary intervention was also terminated early due to severe allergic reactions in 10 of 1605 patients.[203] Acute severe allergic reactions to pegnivacogin were observed exclusively in patients with pre-existing anti-PEG antibodies, and the level of anti-PEG IgG antibodies was associated with the severity of the adverse events.[204] Hypersensitivity reactions can also occur at very low frequencies to PEG administered orally as bowel preparation solution for colonoscopy[205−210] as well as in other medicines and health care products.[211]

Some exogenous peptide and protein drugs failed clinical trials due to unexpected toxicity, but the link to anti-PEG antibodies is unclear. Peginesatide is a synthetic, pegylated dimeric peptide comprised of two identical, 21-amino-acid chains covalently linked to a single branched $PEG_{40\,000}$ molecule for the treatment of anemia in adults with chronic kidney disease receiving hemodialysis.[212] Peginesatide was voluntarily withdrawn from the market after some patients developed severe side effects, including hypotension and anaphylaxis.[213] Antibodies against PEG are recognized as a possible cause of the severe side effects associated with peginesatide, but this link remains unproven.[212]

The mechanism by which anti-PEG antibodies induce hypersensitivity reactions is still unclear. Some possible mechanisms by which pegylated nanoparticles and pegylated nucleotides could induce hypersensitivity reactions are illustrated in Figure 13. Most pre-clinical studies of liposome-induced anaphylaxis have focused on complement activation in a process termed complement activation-related pseudoallergy (CARPA).[15,214−216] IgM or IgG antibodies bound to PEG on a nanoparticle or liposome surface can activate the complement cascade, which liberates the anaphylatoxins C3a and C5a.[15,24,189] A strong link between anti-PEG antibodies and induction of hypersensitivity reactions was recently demonstrated in pigs. Infusion of empty pegylated liposomes induced high levels of anti-PEG IgM, complement activation, and symptoms of anaphylactoid shock immediately after injection of a second dose of pegylated liposomes.[115] However, the clinical relevance of CARPA in the induction of hypersensitivity to pegylated nanomedicines in humans is complicated by large differences in allergic sensitivity between humans and pigs as well as the apparent lack of resident pulmonary intravascular macrophages in humans, which are responsible for the observed hypersensitivity reactions in pigs.[217,218] The clinical significance of complement activation by anti-PEG antibodies in hypersensitivity reactions to nanomedicines therefore requires further investigation.

Immune complexes formed between anti-PEG antibodies and pegylated medicines may also induce hypersensitivity reactions *via* Fc receptor activation of innate immune cells. Classically, anaphylaxis is induced when allergens cross-link immunoglobulin E (IgE) antibodies bound by activating Fc epsilon receptors (FcεRI) on mast cells or basophils, which then rapidly release histamine. Indeed, anti-PEG IgE antibodies are implicated in some hypersensitivity reactions to PEG.[209,219,220] Allergen-specific IgG that form immune complexes can also bind to Fc gamma receptors (FcγRs) expressed on platelets, macrophages, basophils, and neutrophils to release various mediators such as platelet-activating factor (PAF), cysteinyl leukotrienes (CysLTs), histamine, and serotonin.[221−223] Histamine stimulates vasodilation and increases vascular permeability, heart rate, cardiac contraction, and glandular secretion. Serotonin can cause constriction of large blood vessels, capillary dilatation, increased vascular

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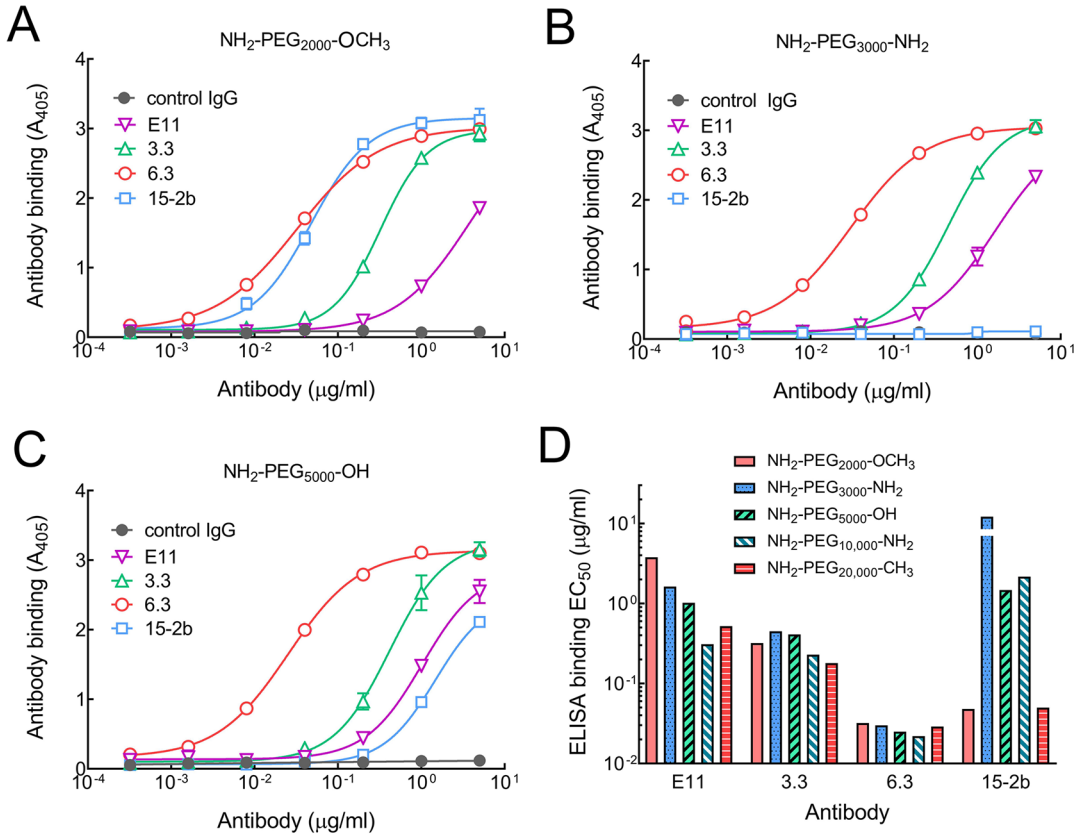

Figure 14. Examples of anti-PEG antibody binding to immobilized PEG. Anti-PEG IgG antibodies E11, 3.3, and 6.3, or anti-mPEG antibody 15-2b, were incubated in 96-well microtiter plates coated with (A) NH_2-PEG_{2000}-O-CH_3, (B) NH_2-PEG_{3000}-NH_2, or (C) NH_2-PEG_{5000}-OH. Binding of the antibodies was determined by addition of horseradish peroxidase-conjugated secondary antibody. Results show the mean absorbance at 405 nm of converted substrate (n = 3). Mean values of triplicate determinations are shown. Bars show SD. (D) The concentrations of each anti-PEG antibody giving 50% maximal response (EC_{50}) in the ELISA assay against the indicated immobilized PEG molecules are shown. The value for 15-2b binding to NH_2-PEG_{3000}-NH_2 is off scale.

permeability, and smooth muscle contraction. PAF can cause bronchoconstriction, platelets aggregation and blood vessels dilation, drop in blood pressure, and reduced volume of blood pumped by the heart. Cysteinyl leukotrienes mediate inflammation, bronchoconstriction, and vascular leakage. The possibility that anti-PEG IgG antibodies are important for hypersensitivity reactions to pegylated drugs is supported by the finding that hypersensitivity reactions induced in mice by pegylated liposomes that encapsulate plasmid DNA could be inhibited by PAF antagonists but was not associated with elevated complement activation.[195] A role was also proposed for Fcγ receptor mediated activation of innate immune cells for hypersensitivity reactions observed during RIG1 therapy due to binding of FcγRs and internalization of pegnivacogin/anti-PEG immune complexes into innate immune cells, resulting in enhanced TLR activation by the RNA aptamer portion of REG1 to enhance allergic responses.[204] Although a role for anti-PEG antibodies in the induction of hypersensitivity reactions to some pegylated medicines is clear, more work is required to define the mechanisms.

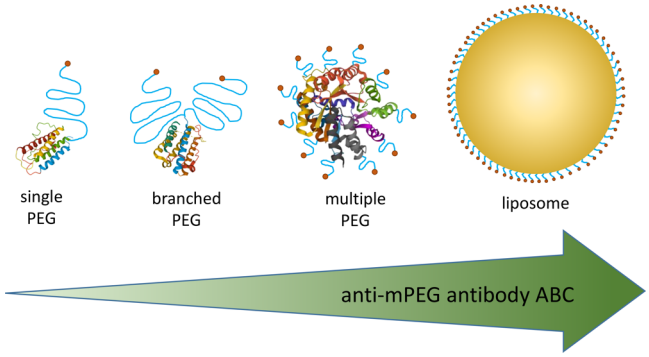

Figure 15. Predicted effect of antibodies against mPEG. Because each PEG chain possesses only one terminal methoxy moiety, immune complex size and biological effects such as ABC may be minimal for drugs with one or two PEG chains but may approach the effect of anti-PEG antibodies for proteins and nanoparticles displaying multiple mPEG binding sites. Blue lines represent the PEG backbone, and red circles represent the terminal methoxy group. Structure images were created using the PDB files 1BUY, 7E0E, and 1VFL with the Mol* Viewer.[38]

BINDING SPECIFICITY OF ANTI-PEG ANTIBODIES

Anti-PEG antibodies can be divided into two groups based on their binding specificity: those that bind to the repeating ethylene oxide subunits or "backbone" of PEG and those that have selectivity for the terminal methoxy group of PEG. We refer to these as anti-PEG or anti-mPEG antibodies, respectively. Specificity for PEG is antibody-dependent with some commercially available anti-PEG antibodies able to bind other polymers such as polypropylene glycol and polytetramethylene ether, whereas others, such as 6.3 and AGP4, display high specificity for PEG.[224] Likewise, some anti-mPEG

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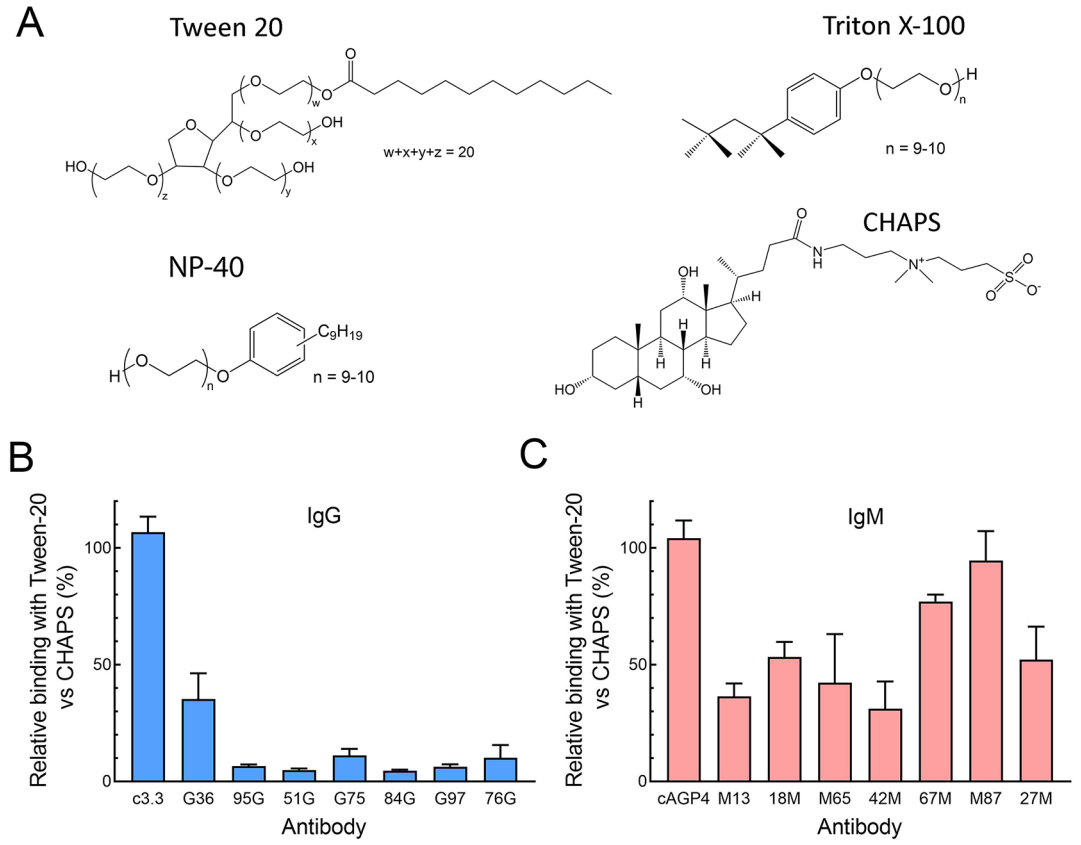

Figure 16. Common detergents can affect anti-PEG antibody binding. (A) Tween-20, Triton X-100, and NP-40 contain ethylene oxide repeats and can be bound by some anti-PEG antibodies. CHAPS is a better alternative detergent for assays using anti-PEG antibodies. Human serum samples that were positive for anti-PEG IgG (B) or anti-PEG IgM (C) antibodies were assayed for binding to NH_2-$PEG_{10\,000}$-NH_2 coated in ELISA plates. Plates were washed between antibody additions with PBS containing 0.05% CHAPS or 0.05% Tween-20 detergent. Human chimeric c3.3 and cAGP4 antibodies, which are resistant to competition with Tween-20, were used as anti-PEG IgG and IgM positive controls, respectively.

antibodies can bind to PEG terminated with chemical moieties that are distinct from a methoxy group such as terminal *n*-butyl ether or monoethyl ether groups.[225,226]

Some of these features are illustrated in Figure 14, in which the binding of selected monoclonal anti-PEG IgG antibodies (E11, 3.3, and 6.3) and one anti-mPEG antibody (15-2b) to immobilized amino-PEG molecules is measured by direct ELISA. Amine-terminated PEG molecules stably adsorb to high-protein binding ELISA plates.[21] All the antibodies bind to NH_2-PEG_{2000}-OCH_3 (methoxy-terminated amino-PEG with molecular weight 2000 Da) (Figure 14A), with 6.3 and 15-2b displaying the highest avidities. 15-2b, by contrast, does not bind NH_2-PEG_{3000}-NH_2 (Figure 14B) and binds to NH_2-PEG_{5000}-OH (Figure 14C) with much lower avidity due to the lack of a terminal methoxy group in these molecules. Comparison of the avidities of these antibodies to immobilized PEG molecules shows that binding of 6.3 and 3.3 antibodies does not depend on the terminal group or PEG length, whereas E11 binds more strongly to longer PEG molecules (Figure 14D). Anti-mPEG antibody 15-2b binding to immobilized PEG varies by over 2 orders of magnitude, mostly depending on the terminal moiety on the PEG chain.

The clinical relevance of anti-mPEG antibodies remains largely unexplored, even though all clinically used pegylated drugs use mPEG. The terminal methoxy group is highly immunogenic in some animal models. Antibodies induced by immunization of rabbits with proteins conjugated with mPEG displayed more than 1000-fold greater affinity to mPEG-protein conjugates as compared to HO-PEG-protein conjugates.[225] Comparison of mPEG and HO-PEG liposomes demonstrated that mPEG generated more anti-PEG IgM antibodies than OH-PEG.[188] The use of HO-PEG instead of mPEG for pegylation has been suggested as a method to produce fewer and less intense immune responses in the clinic.[226] On the other hand, anti-mPEG antibodies were not detected in patients treated with pegloticase, even though 13 patients developed antibodies that bound to the repeating ethylene oxide backbone of PEG.[6] In addition, HO-PEG-coated liposomes activate more complement and experience greater ABC upon a second dose as compared to mPEG-coated liposomes.[188] Antibodies against mPEG may also create smaller immune complexes for some pegylated medicines as compared to anti-PEG antibodies (Figure 15), which should induce less ABC. Additional studies are warranted to accurately assess the clinical impact of anti-mPEG antibodies.

DETERGENTS CAN AFFECT ANTI-PEG ANTIBODY BINDING

Some detergents that are commonly used in immunoassays contain PEG-like domains (Figure 16A) and can interfere with anti-PEG antibody binding.[225] This is illustrated for Tween-20, which is commonly used for washing steps in ELISA. Antibodies present in serum samples from normal donors previously identified as positive for human anti-PEG antibod-

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ies[21] were assayed by direct ELISA for binding to immobilized $PEG_{10\,000}$. The assay was carried out using 0.05% Tween-20 or 0.05% CHAPS, which does not contain ethylene oxide repeats, in the wash buffers. The binding of most human IgG antibodies is decreased by up to 90% when plates are washed with buffer containing Tween-20 as compared to CHAPS (Figure 16B). Anti-PEG IgM antibodies are less affected by the presence of Tween-20 in the wash buffer, probably due to the multivalent binding of IgM to immobilized PEG molecules (Figure 16C). The common use of Tween-20 and similar detergents in ELISA assays may have caused under-reporting of anti-PEG IgG antibodies in many studies.[110,111,121,124−127,131,160,164,169,227,228] Not every antibody is negatively affected by Tween-20, as can be seen for human chimeric c3.3 and cAGP4 antibodies used as antibody standards in the assay.[21] Even though Tween-20 only possesses short stretches of ethylene oxide repeats, the large molar excess of detergent (~0.45 mM) as compared to anti-PEG antibodies (~5 nM) or pegylated compounds (<1 nM) appears to be sufficient for effective competition of some anti-PEG antibodies in ELISA.

PEG SIZE AND IMMOBILIZATION STATUS AFFECT ANTI-PEG ANTIBODY BINDING

Most anti-PEG antibodies bind well to immobilized PEG with molecular weights of 2000 Da or larger. Binding to shorter PEG is antibody-dependent, with some anti-PEG IgM antibodies able to bind very short PEG molecules encompassing only a few ethylene oxide repeats. Insight into how anti-PEG antibodies bind PEG is recently available from the crystal structures of two anti-PEG monoclonal antibodies (3.3 and 6.3).[229,230] Surprisingly, in both cases, two symmetry-related Fab domains cooperate to form an extended PEG binding site. In 3.3, two Fab fragments present on two separate anti-PEG antibodies bind an S-shaped core PEG fragment of about 550 Da, corresponding to about 12 ethylene oxide subunits, by making extensive contacts with aromatic amino acids on the antibody heavy chain and forming hydrogen bonds between a water molecule and the ether oxygen in the PEG backbone.[229] Amino acids present on both the heavy- and light-chain variable region contribute to an additional satellite binding site which can accommodate parts of longer PEG molecules, consistent with enhanced anti-PEG antibody binding to PEG molecules of about 2000 Da or greater. The PEG binding site in 6.3, by contrast, represents a more three-dimensional and dynamic structure in which PEG forms a spiral shape around a tryptophan reside in the heavy chain and then contacts additional amino acids in both the light- and heavy-chain variable regions.[230] The primary PEG epitope corresponds to 16 ethylene oxide subunits (~700 Da) with binding energy coming primarily from van der Waals interactions and burying of surface area.

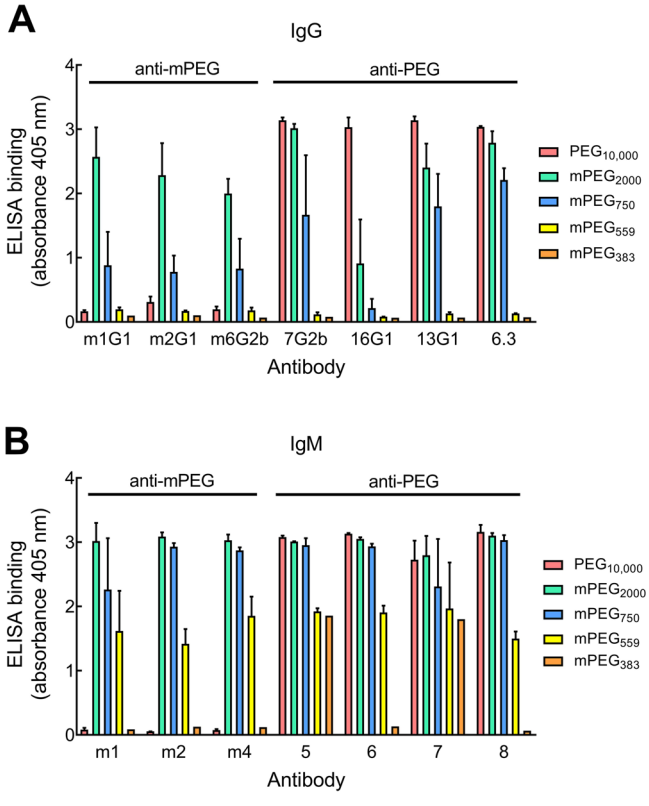

Figure 17. Binding of antibodies to immobilized short PEG molecules. (A) Mouse monoclonal anti-mPEG or anti-PEG IgG antibodies or (B) rat monoclonal anti-mPEG or anti-PEG IgM antibodies from individual hybridoma clones were assessed by direct ELISA for binding to immobilized NH_2-$PEG_{10\,000}$-NH_2 or NH_2-mPEG molecules with molecular weights of 2000, 750, 559, or 383 Da. Results show mean values of duplicate or triplicate determinations. Bars show SD.

To illustrate how PEG length impacts anti-PEG antibodies, binding of a random selection of monoclonal anti-PEG and anti-mPEG antibodies to immobilized $PEG_{10\,000}$ or mPEG ranging in size from $mPEG_{383}$ to $mPEG_{2000}$ was measured by direct ELISA (Figure 17). $mPEG_{383}$ contains 8 ethylene oxide repeats, $mPEG_{559}$ contains 12 repeats, and $mPEG_{750}$ contains 17 repeats. The anti-mPEG IgG antibodies displayed poor binding to $mPEG_{750}$ and shorter mPEG molecules, whereas several anti-PEG IgG clones bound $mPEG_{750}$ but not smaller mPEG molecules (Figure 17A). The anti-mPEG IgM clones all displayed binding to $mPEG_{559}$ and longer mPEG chains (Figure 17B). All anti-PEG IgM clones bound to $mPEG_{559}$, and two clones bound to $mPEG_{383}$. None of the anti-mPEG IgG or IgM clones bound to immobilized $PEG_{10\,000}$, which does not have a terminal methoxy group. These results are typical of a large number of monoclonal antibodies we have examined. In general, anti-mPEG antibodies tend to bind poorly to very short mPEG molecules while anti-PEG IgM is able to bind shorter PEG molecules as compared to anti-PEG IgG, likely due to cooperative binding to multiple PEG molecules. Rats seem to produce more IgM antibodies with specificity to mPEG as compared to mice.

The sensitivity by which PEGylated compounds or PEG molecules in solution can be detected with anti-PEG antibodies also depends on PEG size. Comparison of the detection of pegylated compounds in a sandwich ELISA shows that compounds with longer PEG chains (Pegasys has a single branched $PEG_{40\,000}$, and Mircera has a single $PEG_{30,000}$) are detected at picomolar levels of PEG, whereas compounds with shorter PEG chains (Lipodox and qdots have multiple PEG_{2000} molecules attached to their surface) are detected at nanomolar concentrations of PEG (Figure 18A). This sandwich assay uses immobilized rAGP6 anti-PEG IgM antibody to capture the pegylated compounds, which are then detected by biotinylated 6.3 anti-PEG IgG (6.3-biotin).

Short PEG molecules in solution that are not immobilized on one end (e.g., attached to an ELISA plate, protein,

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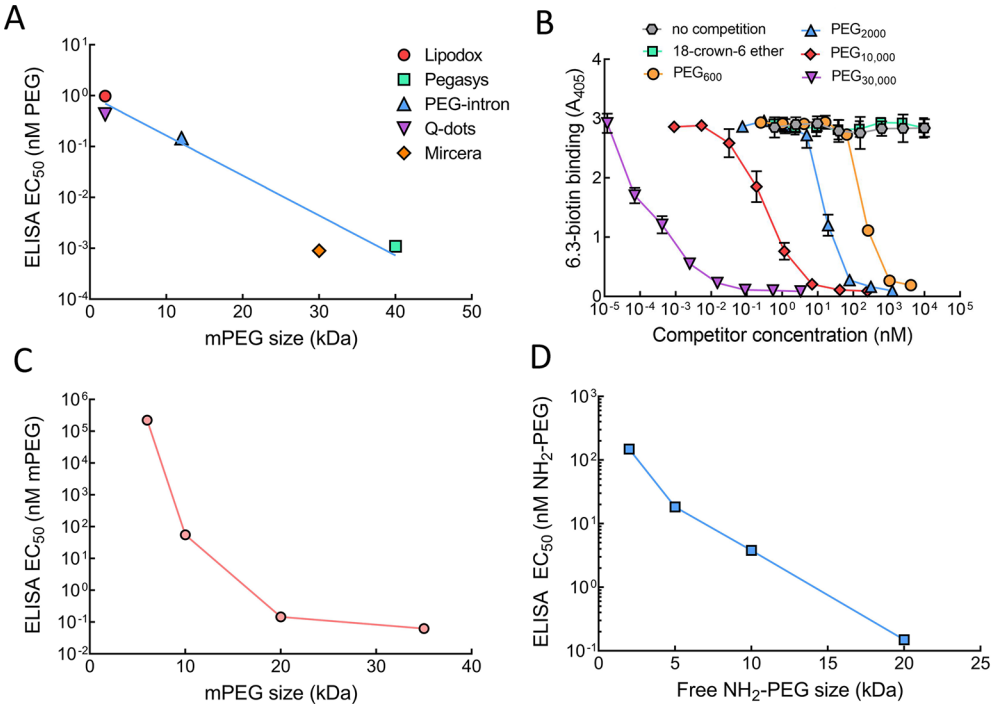

Figure 18. Sandwich ELISA detection of PEG-modified drugs or free PEG molecules. (A) EC_{50} values based on the concentration of PEG in PEG-modified compounds as measured by sandwich ELISA using rAGP6 for capture and 6.3-biotin for detection. (B) Competition of the binding of 6.3-biotin to immobilized NH_2-$PEG_{10\,000}$ with the indicated free PEG or PEG-like molecules. (C) EC_{50} values for the assay of soluble PEG molecules by sandwich ELISA with rAGP6 and 6.3-biotin antibodies. (D) EC_{50} values for the assay of soluble amine-PEG molecules by sandwich ELISA with rAGP6 and 6.3-biotin antibodies.

liposome, or nanoparticle) are poorly recognized by anti-PEG antibodies. For example, nearly micromolar concentrations of PEG_{600} are required to compete 6.3-boiotin binding to immobilized amino-$PEG_{10\,000}$ (Figure 18B). By contrast, longer soluble PEG molecules can effectively compete 6.3-biotin binding to immobilized PEG; $PEG_{30\,000}$ competes binding of 6.3-biotin antibody at picomolar concentrations, almost 7 orders of magnitude lower concentrations than PEG_{600} (Figure 18B). Similar strong dependence on the size of non-immobilized PEG is also observed in a sandwich assay using rAGP6 for capture and 6.3-biotin for detection with sensitivities ranging over 10 million-fold for soluble $PEG_{35\,000}$ *versus* PEG_{6000} (Figure 18C). In our experience, soluble PEG of less than about 4000 Da is almost undetectable by sandwich ELISA. On the other hand, small amine-functionalized PEG molecules can be detected with relatively good sensitivity (Figure 18D), likely due to interaction of the amine group with surfaces or proteins to provide an immobilized-like PEG molecule.

CONCLUDING REMARKS

It is easy to either overstate or downplay issues related to PEG immunogenicity. A clear understanding of when and how anti-PEG antibodies impact drug efficacy and safety is needed for a realistic assessment of one of the most widely used and successful polymers in drug delivery. Accelerated blood clearance, which primarily decreases therapeutic efficacy, is a potential issue for pegylated drugs administered at low doses because there is a greater likelihood that molar anti-PEG antibody concentration exceeds drug concentration and immune complexes can form (Figure 19). On the other hand, hypersensitivity reactions are more likely to occur when

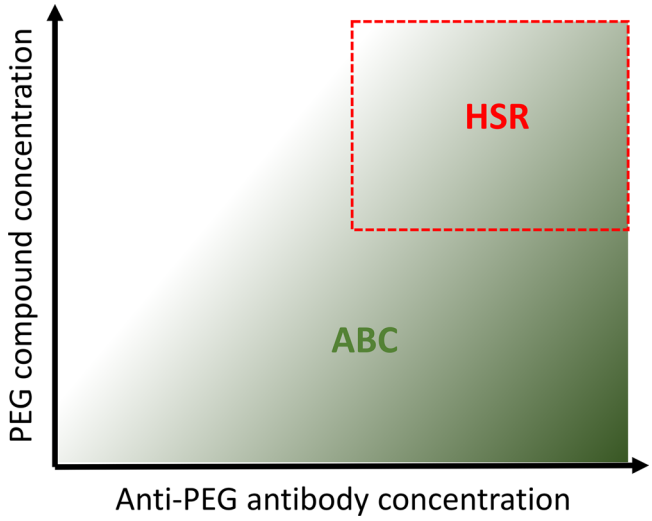

Figure 19. Regimes of anti-PEG antibody impact on pegylated medicines. ABC (green) is more problematic for pegylated compounds administered at low doses, whereas hypersensitivity reactions (dotted red box) are more likely to occur when both pegylated drug and anti-PEG antibody concentrations are high.

both anti-PEG antibody and drug concentrations are relatively high because sufficient complement and/or innate immune cells are activated to cause safety issues. Drugs that can directly activate the immune system move the window to lower anti-PEG antibody concentrations. Although it is now clear that anti-PEG antibodies can induce hypersensitivity reactions, the mechanisms in humans remain unclear. It is important to define the pathways responsible for induction of hypersensitivity to pegylated medicines to develop rational medical

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Table 6. Examples of PEG Alternatives

technology	material	type	applications	status	citation
PASylation	Pro, Ala, and Ser polypeptide	disordered polypeptide	proteins	pre-clinical	235
PMeOx	poly(2-oxazoline)	amphipathic polymer	proteins, liposomes, nanoparticles	pre-clinical, early clinical	236
PCB	poly(carboxybetaine)	zwitterionic polymer	proteins, liposomes, nanoparticles	pre-clinical	237
XTEN	Ala, Asp, Gly, Pro, Ser, and Thr polypeptide	unstructured polypeptide	proteins	early clinical	238
Fc fusion	immunoglobulin Fc domain	protein domain	proteins	clinical approval	239

interventions and increase patient safety. A role for IgE antibodies against PEG in allergic reactions warrants further investigation.[220]

Pre-existing antibodies that bind PEG are common in the general population, but the antibodies are present at concentrations that do not affect drug safety or efficacy in most individuals. However, pegylated cytokines such as the interferons, which are administered at microgram doses, may undergo ABC caused by anti-PEG antibodies. Micelles and proteins administered at relatively high molar doses do not normally experience ABC because insufficient pre-existing anti-PEG antibodies are present to form immune complexes (anti-PEG antibody ≪ drug). Special care should be paid to pegylated compounds which can directly activate innate immune cells, such as aptamers which bind to TLRs, because they may induce serious hypersensitivity reactions in some patients with pre-existing antibodies against PEG.[52,202−204] It is strongly suggested to screen volunteers or patients for anti-PEG antibody levels before initiation of clinical trials of pegylated medicines to ensure patient safety and to minimize the chance of study failure.

Induction of antibodies against PEG occurs by the thymus-dependent pathway for polypeptides and by the TI-2 pathway for non-protein compounds. The magnitude of the anti-PEG antibody response by either pathway primarily depends on the immunogenicity of the non-PEG portion of the compound. Thus, totally human proteins do not induce anti-PEG antibodies unless the patient lacks the protein such as in factor VIII replacement therapy for treatment of severe hemophilia. Non-human proteins universally generate anti-PEG antibodies. Reduction of protein immunogenicity by removal of MHC binding epitopes, directed evolution of human analogs, or development of human antibodies to carry out the missing function may be promising approaches to reduce anti-PEG antibody responses.[104,231−233] Induction of anti-PEG antibodies against nanomedicines depends on the immunogenicity of the payload. Cytotoxic drugs do not usually induce anti-PEG antibodies because anti-PEG B cells are selectively killed. Encapsulation of non-modified DNA or RNA molecules, on the other hand, may generate strong antibody responses against PEG.

The elephant in the PEG room is the widespread use of SARS-CoV-2 RNA vaccines. Important questions remain to be answered, including how many people receiving BNT162b or mRNA-1273 develop antibodies against PEG, how long induced anti-PEG antibodies remain in the circulation, and whether memory B cell responses are generated. It is critical that physicians are made aware that the safety and efficacy of previously safe pegylated medicines may change, especially if booster vaccinations of SARS-CoV-2 RNA vaccines are necessary, which seems increasingly likely as more SARS-CoV-2 variants emerge.[234] At a minimum, more testing for anti-PEG antibodies before administration of pegylated drugs may be warranted. Widespread use of RNA vaccines may accelerate development of PEG alternatives (Table 6).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.1c05922.

Pre-existing anti-PEG antibody assay; incidence and concentrations of pre-existing human anti-PEG antibodies in healthy donors; sex and age distributions of 2404 donors in study of pre-existing antibodies against PEG; accelerated blood clearance of nanoparticles containing cytotoxic drugs; relationship between accelerated blood clearance and anti-PEG antibody/drug ratio; estimated concentration of pre-existing anti-PEG antibodies to exceed pegylated drug number by 10-fold (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors (B.M. Chen, T. L. Cheng and S.R. Roffler) may benefit from the licensing or commercial transfer of anti-PEG antibodies developed in the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan and Kaohsiung Medical School, Kaohsiung, Taiwan (https://www.ibms.sinica.edu.tw/~sroff/anti-PEG/index.html).

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VOCABULARY

accelerated blood clearance (ABC), a phenomenon in which drugs are cleared from the circulation at an abnormally fast rate; **pegylation**, physical attachment of PEG to a therapeutic

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molecule, usually a peptide, protein, nucleic acid, liposome, or nanoparticle, to reduce administration frequency or therapeutic efficacy; **toll-like receptor (TLR)**, a family of surface receptors that sense pathogens and molecular danger signas to initiate inflammatory responses; **thymus-dependent (TD) antigen**, a polypeptide that can generate antibody responses with help from T cells; **thymus-independent type-2 (TI-2) antigen**, a polyvalent antigen that can elicit antibody responses without T cell help

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