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ARTICLE

Antibodies against Poly(ethylene glycol) Activate Innate Immune Cells and Induce Hypersensitivity Reactions to PEGylated Nanomedicines

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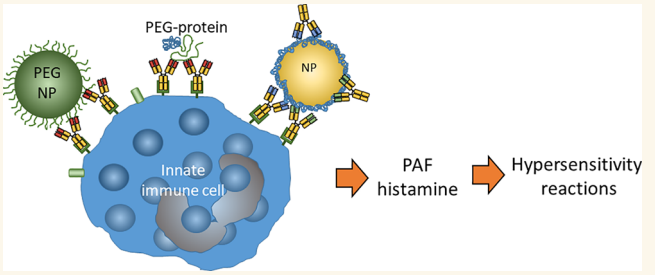
ABSTRACT: **Nanomedicines and macromolecular drugs can induce hypersensitivity reactions (HSRs) with symptoms ranging from flushing and breathing difficulties to hypothermia, hypotension, and death in the most severe cases. Because many normal individuals have pre-existing antibodies that bind to poly(ethylene glycol) (PEG), which is often present on the surface of nanomedicines and macromolecular drugs, we examined if and how anti-PEG antibodies induce HSRs to PEGylated liposomal doxorubicin (PLD). Anti-PEG IgG but not anti-PEG IgM induced symptoms of HSRs including hypothermia, altered lung function, and hypotension after PLD administration in C57BL/6 and nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Hypothermia was significantly reduced by blocking FcγRII/III, by depleting basophils, monocytes, neutrophils, or mast cells, and by inhibiting secretion of histamine and platelet-activating factor. Anti-PEG IgG also induced hypothermia in mice after administration of other PEGylated liposomes, nanoparticles, or proteins. Humanized anti-PEG IgG promoted binding of PEGylated nanoparticles to human immune cells and induced secretion of histamine from human basophils in the presence of PLD. Anti-PEG IgE could also induce hypersensitivity reactions in mice after administration of PLD. Our results demonstrate an important role for IgG antibodies in induction of HSRs to PEGylated nanomedicines through interaction with Fcγ receptors on innate immune cells and provide a deeper understanding of HSRs to PEGylated nanoparticles and macromolecular drugs that may facilitate development of safer nanomedicines.**



KEYWORDS: *poly(ethylene glycol), immunogenicity, anti-PEG antibodies, hypersensitivity reactions, PEGylated liposomal doxorubicin, innate immune cells, basophils*

INTRODUCTION

PEGylated nanoparticles and macromolecular drugs can induce infusion-related hypersensitivity reactions (HSRs) in some patients, particularly during their first exposure to the medicine.[1−3] HSRs usually occur immediately after administration of PEGylated nanomedicines and biomolecules with symptoms that include flushing and facial swelling, breathing difficulties, head and back pain, tightness in the chest or throat, hypothermia, hypotension, and death in the most severe cases.[4] This is typified by PEGylated liposomal doxorubicin (PLD), a liposomal formulation of doxorubicin hydrochloride (Doxil, Caelyx, and their generic versions) used in almost one million cancer patients but which can cause infusion related hypersensitivity reactions (HSRs) in 5% to 10% of patients.[5−7] HSRs can force discontinuation of beneficial treatment, place a substantial burden on the medical system, and may prevent successful translation of PEGylated nanomedicines to the clinic. HSRs are therefore recognized as a major barrier to the development of nanomedicines.[8,9]

HSRs to nanomedicines have been linked to complement activation-related pseudoallergy (CARPA), in which liberation of complement products stimulate innate immune cells to secrete vasoactive and inflammatory mediators.[6,10] Indeed, the

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levels of plasma C terminal complex (SC5b-9) is dose-dependently increased in patients experiencing HSR.[5,11] Rapid uptake of nanoparticles into phagocytic cells such as macrophages and activation of innate immune cells independently of complement activation have also been proposed to cause infusion related adverse reactions to nanoparticles.[12,13] However, the actual mechanism of HSRs caused by nanomedicines remains controversial.[3,13,14]

Poly(ethylene glycol) (PEG) is physically attached to many macromolecular drugs, nanoparticles, and liposomes to achieve desirable pharmacokinetic properties and enhance their biological activity. Antibodies against PEG, however, are naturally present in many normal individuals, possibly due to exposure to PEG in a wide range of household products such as lotions, creams, and shampoos, which is why they are also referred to as pre-existing anti-PEG antibodies.[15,16] Some PEGylated drugs also induce the production of antibodies against PEG.[17−20] Anti-PEG antibodies can accelerate drug clearance from the circulation, alter drug biodistribution, activate complement, destabilize the integrity of PEGylated nanomedicines, and reduce drug therapeutic efficacy.[21−27] Anti-PEG antibodies can also induce hypersensitivity reactions in patients receiving PEGylated medicines.[5,6,28,29]

Here we examine the hypothesis that anti-PEG antibodies bound to PEGylated nanoparticles and macromolecules can interact with Fc receptors on immune cells to initiate HSRs.[30] We demonstrate that anti-PEG IgG but not IgM antibodies induce hypersensitivity-like symptoms against PLD and other PEGylated nanoparticles and macromolecules in mice that depend primarily on neutrophils, macrophages, and basophils. HSR symptoms are alleviated by blocking Fcγ receptors and elaboration of histamine and platelet-activating factor. Human anti-PEG IgG can induce histamine secretion from human basophils in the presence of PLD, consistent with an important role for Fcγ receptor-mediated responses to antibodies bound to PEGylated nanoparticles in HSRs.

MATERIALS AND METHODS

Animals. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prkdcscid/NcrCrl, 8−12 weeks old) were obtained from BioLASCO Taiwan Co., Ltd. BALB/c and C57BL/6JNarl mice (8−12 weeks old) were obtained from the National Laboratory Animal Center, Taipei, Taiwan. Sash (c-Kit$^{w\text{-}sh}$) mice were a kind gift from Dr. Ya-Jen Chang (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions. All animal experiments were performed according to institutional guidelines and ethically approved by the Laboratory Animal Facility and Pathology Core Committee of IBMS, Academia Sinica.

Reagents. PEGylated liposomal doxorubicin (Lipo-Dox, PLD) was from Taiwan Tung Yang Biopharm (TTY Biopharm Company Ltd., Taipei, Taiwan). We previously demonstrated that these PLD particles are identical to Doxil in their interaction with anti-PEG antibodies.[58] Onivyde (irinotecan liposomal, IL) was provided by Kaohsiung Medical University (Kaohsiung, Taiwan). CV6209, *N*-formylmethionyl-leucyl-phenylalanine (fMLF), A23187, cetirizine, ranitidine, RPMI 1640, PEGylated Fe_3O_4 nanoparticles (NPs) (30 nm average, −OH terminal, 2000 PEG), and 2,4-dinitrophenyl conjugated human serum albumin (DNP-HSA) were from Sigma-Aldrich (St Louis, Mo, USA). Qdot 655 ITK Amino (PEG) Quantum Dots were from Thermo Fisher Scientific (Waltham, Massachusetts). BSA-PEG_{13} was generated by reacting 10.4 mg PEG_{5000} succinimidyl ester (NANOCS, Boston, MA) with 5 mg bovine serum albumin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 2 h at room temperature in 0.1 M sodium tetraborate, 50 mM NaCl, pH 8, before unreacted PEG was removed by gel filtration on a 1.6 × 100 cm^2 Sephacryl S-300 HR column equilibrated with PBS. Liposomal clodronate was purchased from Encapsula NanoSciences (Brentwood, TN, USA). MidiMacs separation columns, MidiMacs magnetic columns, and MACSxpress separator were from Miltenyi Biotec (Bergisch Gladbach, Germany). ELISA kits for histamine were from LDN (Nordhorn, Germany). Mouse MCPT-1 (mast cell protease 1) BT-Lab ELISA was from Korain Biotech Co (Jiaxing, China).

Antibodies. Antibodies used in this work are listed in Supplemental Table 1. Mouse anti-PEG IgG (6.3, 3.3) and IgM (AGP4) antibodies and mouse anti-mPEG antibody 15-2b have been previously described.[31−33] Humanized 3.3 (c3.3-IgG) and 6.3 (Hu-6.3) anti-PEG antibodies have been described.[15,25] 15-2c anti-mPEG antibody and 3.3-IgE anti-PEG antibodies were generated by CRISPR-Cas9 mediated class switch recombination in the 15-2b and 3.3 hybridoma cells, respectively.[34] Control mouse IgG_1, IgG_{2b}, and IgM antibodies were purified from hybridoma cells. All anti-PEG antibodies were purified by ion-exchange on diethylaminoethyl cellulose (DEAE) to minimize aggregation. Anti-DNP IgE was a kind gift from Dr. Fu-Tong Liu (Vice President, Academia Sinica, Taipei, Taiwan). RB6-8C5 (rat anti-mouse Ly6G), 6S2-19-4 (rat anti-mouse CCR3/CD193), 2.4G2 (rat anti-mouse CD16/CD32), and isotype-matched control antibodies were obtained from Bio X Cell (West Lebanon, NH). Ba103 (rat anti-mouse CD200R3) was purchased from Hycult Biotech (The Netherlands). ACK2 rat anti-mouse c-kit antibody was purified from the culture supernatant of RCB4477:ACK2 hybridoma cells obtained from the RIKEN BioResource Research Center Cell Bank (Japan). Aggregated mouse IgG, purified from mouse serum, was generated by incubation at 25 mg mL^{-1} in borate-buffered saline [0.17 M H_3BO_3 and 0.12 M NaCl (pH 8)] for 1 h at 63 °C and then diluted to 10 mg mL^{-1} in 0.9% NaCl.

Anti-PEG Antibody Binding Assay. Anti-PEG antibody binding to liposomes was examined by modification of a previously published method.[32] EIA microplates were coated overnight with 0.75 μg per well of 15-2b (anti-mPEG IgG) in PBS. Plates were washed once with PBS and then blocked with 5% skim milk powder in PBS. PLD and IL were diluted to 20 μg mL^{-1} (total lipid concentration) in 2% skim milk/PBS, then serially diluted five times in 2% skim milk/PBS before 50 μL aliquots were added to the plate. After 60 min at room temperature, excess liposomes were removed by washing with PBS three times before 1 μg mL^{-1} 3.3−biotin, AGP4−biotin, or negative control antibody−biotin was added to the plates for 45 min. After washing three times with PBS, 0.5 μg/well HRP−streptavidin (Jackson Immunoresearch; 016-030-084) was added for 45 min. After washing plates three times with PBS, peroxidase activity was quantified by addition of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and H_2O_2 (3000:1) for 30 min before reading the absorbance at 405 nm.

In Vivo Hypersensitivity Model. Mouse anti-PEG IgG_1 (6.3, 3.3), anti-PEG IgM (AGP4), or control IgG_1 and IgM antibodies were intravenously administered to female NOD/SCID mice aged 8−12 weeks at doses of 2 mg kg^{-1} or 10 mg kg^{-1}, respectively. Aggregated mouse IgG was iv injected at 50 mg kg^{-1} as a positive control. After 1 h, PLD at a dose of 2 mg kg^{-1} was intravenously injected into the mice. For C57BL/6JNarl mice and BALB/c mice, 25 mg kg^{-1} of 3.3, AGP4, or isotype-matched control antibodies were intravenously injected 3 h before iv administration of 2 mg kg^{-1} PLD. The body temperature of mice was monitored with a digital thermometer (Center 301, 374) with rectal probe (Center Technology, Taiwan). Lung function was evaluated by measuring enhanced pause (P_{enh}) of mice by unrestrained whole-body plethysmography (DSI Buxco Inc., USA). Systolic and diastolic blood pressures of mice were monitored and analyzed by BP-2000 Series II Blood Pressure Analysis System (Visitech Systems Inc., USA). In some experiments, the timing and doses of antibodies were varied as indicated in the figure legends. To examine if other PEGylated medicines can induce HSR, mice were injected with 2 mg kg^{-1} 3.3 antibody 1 h before intravenous administration of 12 mg kg^{-1} IL, 6 × 10^{12} iron oxide NPs (IONPs), 0.2 mg kg^{-1} BSA-PEG, or 0.1

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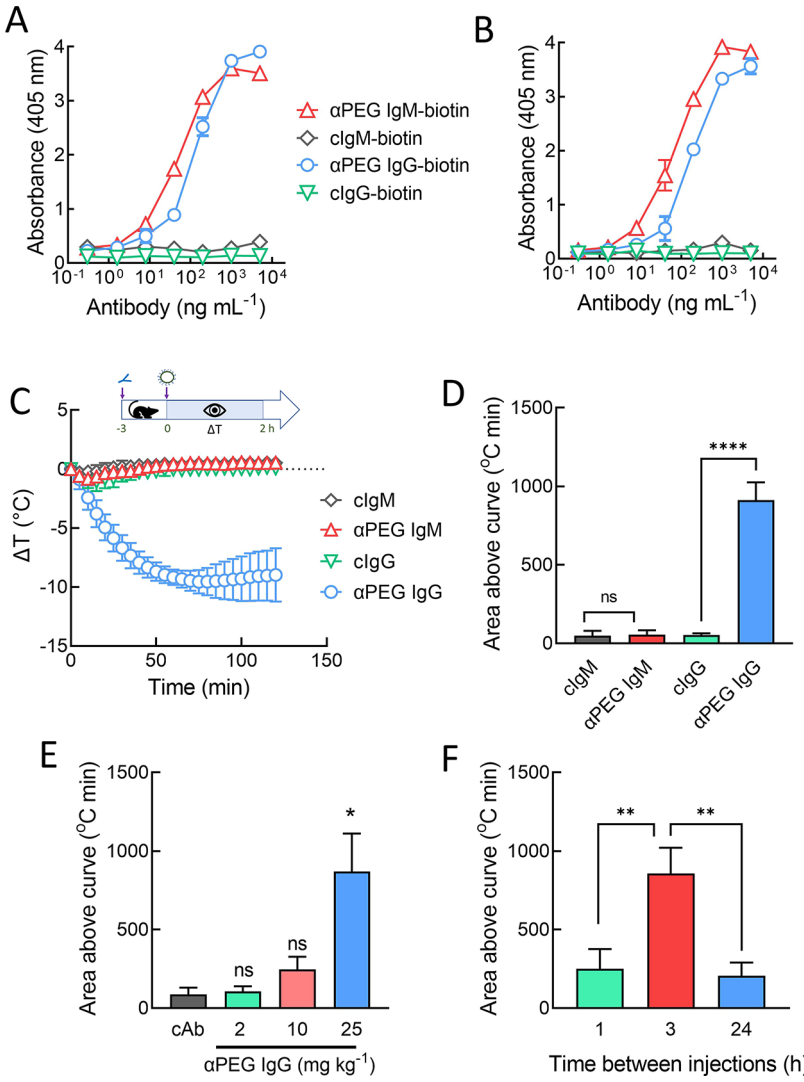

Figure 1. Anti-PEG IgG triggers hypothermia in C57BL/6 mice after systemic administration of PLD. Serial dilutions of biotinylated anti-PEG antibodies or biotin-labeled isotype-matched control antibodies were incubated with immobilized PLD (A) or IL (B) before antibody binding was assessed by HRP−streptavidin in a direct ELISA. Bars, SD; n = 3. (C) C57BL/6JNarl mice were iv injected with 25 mg kg^{-1} anti-PEG IgG (3.3), anti-PEG IgM (AGP4), or isotype-matched control antibodies (cIgG or cIgM) 3 h before iv injection of 2 mg kg^{-1} PLD. Results show mean change in the internal temperature of mice (Bars, SD; n = 5). (D) Mean area above the curves for the results shown in panel C (Bars, SD; n = 5). (E) Mean area under the curves for temperature drops induced by iv injection of the indicated doses of anti-PEG IgG or 25 mg kg^{-1} control IgG 3 h before administration of 2 mg kg^{-1} PLD (Bars, SD; n = 3). (F) Mean area under the curve for temperature drops induced by iv injection of 25 mg kg^{-1} anti-PEG IgG or control IgG 1, 3, or 24 h before administration of 2 mg kg^{-1} PLD (Bars, SD; n = 3). Significant differences between mean values of anti-PEG antibodies versus control antibodies are indicated: *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$; ns, not significant.

mg kg^{-1} BSA, which correspond to similar molar doses as 2 mg kg^{-1} PLD.

In Vivo Receptor Blocking and Cellular Depletion. To block FcγR III/II, 1.2 mg kg^{-1} of 2.4G2 (rat anti-mouse CD16/CD32) or rat IgG$_{2b}$ isotype control were mixed with 2 mg kg^{-1} 3.3 anti-PEG IgG thoroughly and injected intravenously 1 h before iv injection of 2 mg kg^{-1} PLD.[35] To deplete neutrophils, mice were iv injected with 12 mg kg^{-1} rat anti-Ly6G or rat IgG$_{2a}$ isotype control 24 h before challenge with anti-PEG antibodies and PLD.[35] Liver macrophages were depleted by intravenous injection of 150 μL per mouse of liposomal clodronate or PBS 4 h before challenge with anti-PEG antibodies and PLD.[35] To deplete basophils, 2.2 mg kg^{-1} of Ba103 were injected iv 24 h before challenge.[36] To deplete eosinophils, 12 mg kg^{-1} anti-mouse CCR3 antibody was injected intraperitoneally 24 h before challenge.[35] To deplete mast cells, mice were administered five doses (first dose intravenously, then intraperitoneally) of 1 mg of anti-c-kit antibody (ACK2) or an isotype control IgG$_{2b}$ antibody for 5 days before challenge.[37] Mast cell depletion was verified by iv administration of 0.4 mg kg^{-1} anti-DNP IgE antibody 24 h before intravenous challenge with 4 mg kg^{-1} DNP-BSA. The concentration of MCPT-1 was measured by ELISA in blood samples collected 1 h later.[38]

To block histamine receptor type I, type II, or both, 12 mg kg^{-1} cetirizine, 40 mg kg^{-1} ranitidine, or both was intraperitoneally injected 30 and 60 min, respectively, before challenge with anti-PEG antibodies and PLD.[39] Platelet-activating factor (PAF) receptor (PAFR) antagonist CV-6209 (3.3 mg kg^{-1}) was intravenously injected 10 min before challenge with anti-PEG antibodies and PLD.[40]

Binding of PEGylated Quantum Dots to Immune Cells. Anti-PEG nanoparticle immune complexes were formed by incubating 10 μg anti-PEG IgG (3.3 or 6.3) or negative control IgG with 1 μL of Qdot 655 ITK Amino (PEG) Quantum Dots, 8 μM in 400 μL PBS, for 30 min at room temperature. Peripheral blood mononuclear cells

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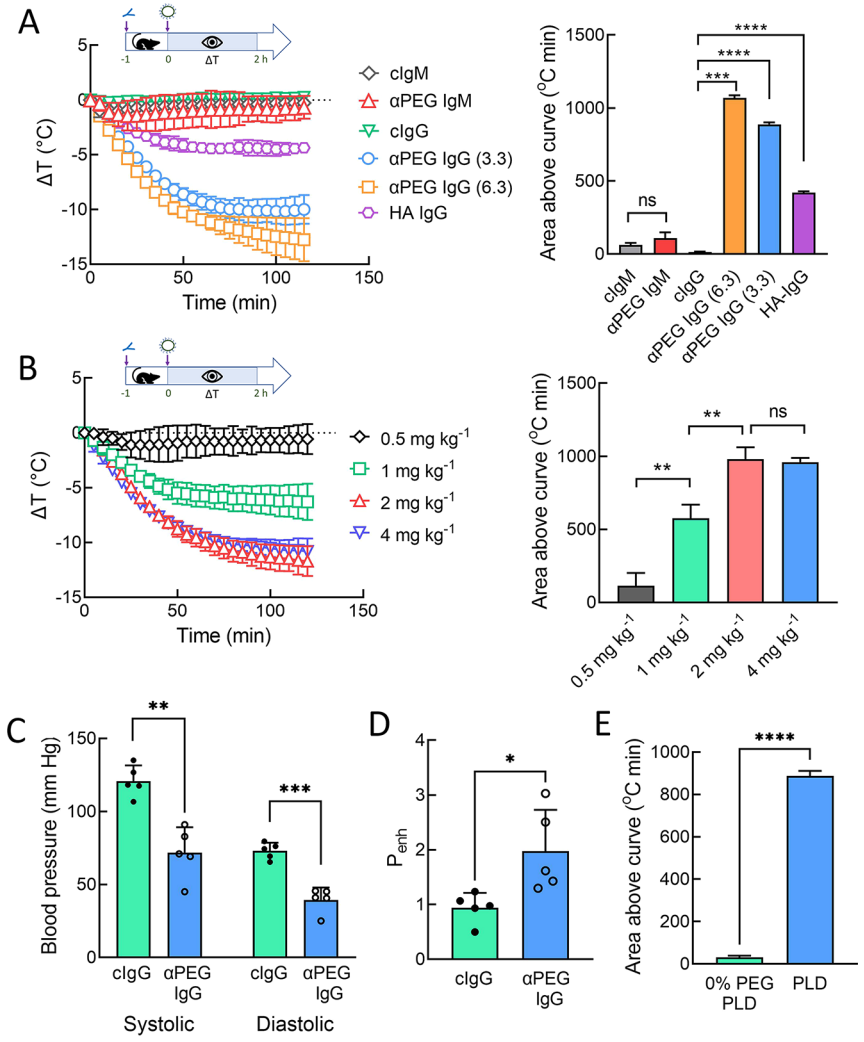

Figure 2. Anti-PEG IgG can induce hypersensitivity reactions in NOD/SCID mice treated with PLD. (A) Mean temperature drop (left) and area above the curves (right) of NOD/SCID mice iv injected with 2 mg kg^{-1} anti-PEG IgG or cIgG, or 10 mg kg^{-1} anti-PEG IgM or cIgM 1 h before iv injection of 2 mg kg^{-1} PLD. Positive control mice were iv injected with 50 mg kg^{-1} heat-aggregated immunoglobulins (HA IgG) (Bars, SD; n = 3). (B) Mean temperature drop (left) and area above the curve (right) in mice treated with the indicated doses of anti-PEG IgG 1 h before injection of PLD (Bars, SD; n = 3). (C) Blood pressure and (D) lung function of mice was analyzed immediately before or 30 min after administration of PLD in mice preteated with 2 mg kg^{-1} anti-PEG IgG or cIgG (Bars, SD; n = 5). (E) Comparison of hypothermia in mice injected with 2 mg kg^{-1} anti-PEG IgG 1 h before iv injection of 2 mg kg^{-1} non-PEGylated LD or PLD (Bars, SD; n = 3). Significant differences between mean values are indicated: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; ns, not significant.

(PBMCs) were isolated from whole blood collected from NOD/SCID mice. Red blood cells were lysed by addition of ACK buffer. PBMCs were incubated with PBS or 2 μg Fc Block per 10^6 cells for 15 min on ice before addition of 200 μL anti-PEG or control IgG–QD655 immune complexes on ice for 30 min. The cells were washed with PBS and suspended in PBS supplemented with propidium iodide to identify dead cells. Live cells that displayed positive QD655 fluorescence were sorted on a BD LSR II flow cytometer, fixed on glass coverslips with methanol, air-dried, and stained with Giemsa Stain solution (Sigma, diluted 1:20 with distilled water) for 60 min. Cell morphology was observed on a Zeiss Imager A1 microscope. Mouse liver Kupffer cells isolated from NOD/SCID mice using a Percoll gradient procedure were allowed to attach on glass coverslips in serum-free DMEM for 30 min at 37 °C.[41] Unattached cells were removed by washing with DMEM and then PBS or 5 μg Fc Block was added to coverslips on ice for 15 min before addition of anti-PEG or control IgG-QD655 immune complex supplemented with 1 μg mL^{-1} Hoechst 33342 dye for 30 min on ice. The slides were washed twice with PBS and then analyzed on a LSM880 confocal microscope at excitation/emission wavelengths of 350/461 nm for DNA stained with Hoechst 33342 dye and 350/675 nm for QD655, respectively.

Human neutrophils were isolated from 8 mL of blood collected from normal donors in EDTA-coated collection tubes by magnetic depletion of nontargeted cells using the MACS–whole blood neutrophils isolation kit. Human basophils were isolated from 100 mL of blood collected from normal donors in EDTA-coated collection tubes by first diluting blood 3.5-fold in PBS containing 0.5% BSA and 2 mM EDTA. Cell suspensions (35 mL) were layered on 15 mL of Ficoll-Paque (ρ = 1.077 g cm^{-3}) in 50 mL tubes and centrifuged for 30 min at 400 × g to isolate mononuclear cells at the interface. The mononuclear cells were washed twice with PBS containing 0.5% BSA and 2 mM EDTA, and then basophils were isolated using the MACS-basophil isolation kit II. Anti-PEG nanoparticle immune complexes were formed by incubating 10 μg c3.3-IgG, Hu6.3, or negative control IgG purified from normal donors with 1 μL of QD655 in 200 μL RPMI 1640 medium supplemented with 10% fetal bovine serum for 30 min at room temperature. Human neutrophils or basophils were incubated with the mixture for 30 min on ice, washed twice with PBS, and then analyzed for bound QD655

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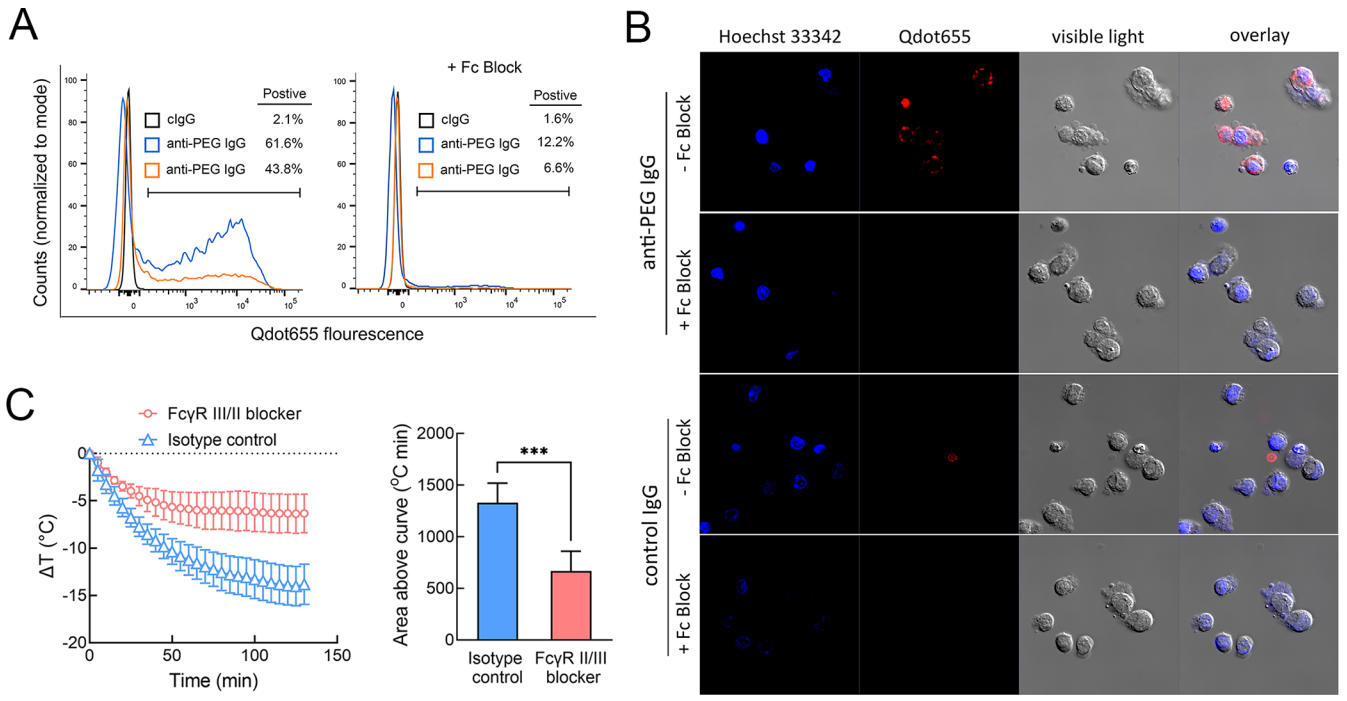

Figure 3. Anti-PEG IgG can cross-link PEGylated nanoparticles and immune cells via FcγII/III receptors. (A) Peripheral blood mononuclear cells from NOD/SCID mice incubated with a mixture of QD655 and anti-PEG IgG (3.3, blue; 6.3, orange) or cIgG without (left) or with Fc Block (right) were analyzed by flow cytometry for QD fluorescence. (B) Kupffer cells from NOD/SCID mice were incubated with QD655 and anti-PEG IgG or cIgG with or without Fc Block. Nuclei were stained with Hoechst 33342 (blue), while red fluorescence indicates QD655. (C) NOD/SCID mice were intravenously injected with 2.4G2 antibody to block FcγII/III receptors along with anti-PEG IgG or cIgG 1 h before injection of 2 mg kg^{-1} PLD. Mean temperature drop (left) and area above the curves (right) are shown (Bars, SD; $n = 5$). Significant differences between mean areas above the curves with and without blocking are indicated: ***$p \leq 0.001$.

fluorescence on a BD LSR II flow cytometer. For monocytes, human PBMCs in serum-free RPMI 1640 were incubated on glass coverslips for 30 min at 37 °C and then gently washed with PBS to preferentially enrich attached monocytes. The slides were incubated for 30 min at 4 °C with the immune complex mixture supplemented with 1 μg mL^{-1} Hoechst 33342 dye. The slides were washed twice with PBS and then analyzed by confocal microscopy as above.

In Vitro Stimulation of Human Basophils. Human basophils were purified from human blood from normal donors as described above. Humanized anti-PEG IgG$_1$ (c3.3-IgG) or anti-PEG IgG$_2$ (c3.3-IgG$_2$), or negative-control human IgG$_1$ or IgG$_2$ (cIgG$_1$ and cIgG$_2$) at concentration of 375 μg mL^{-1} were incubated with 250 μg mL^{-1} PLD in RPMI medium supplemented with 10% fetal bovine serum at 37 °C for 1 h. The mixture was diluted 100-fold, and then further 5-fold serial dilutions were prepared. The antibody−PLD mixtures at initial concentrations of 94 ng mL^{-1} antibody and 62.5 ng mL^{-1} PLD were added to 3 × 10^4 basophils in 24-well plates at 37 °C for 30 min. Control groups included addition of 2.5 μM A23187, 1 μM fMLF, or 1 μg mL^{-1} lipopolysaccharide (LPS).[35] Histamine release in supernatant was quantified by ELISA (BA E-1000 LDN, Germany).

Statistics. All results show mean values ± standard deviation unless otherwise noted. Statistical analyses were performed using GraphPad Prism 9 (La Jolla CA, USA, www.graphpad.com). Significance between mean values of different treatment groups was analyzed using Student's t test with Welch's correction (does not assume equal standard deviations between groups).

RESULTS

Anti-PEG IgG and PLD Can Induce Hypersensitivity-like Reactions in Mice. To investigate possible links between anti-PEG antibodies and PEGylated nanomedicine hypersensitivity, we selected well-characterized anti-PEG IgG (3.3) and anti-PEG IgM (AGP4) antibodies for experiments.[32] Biotinylated anti-PEG IgG (3.3) and IgM (AGP4) antibodies were verified to bind to immobilized PLD (PEGylated liposomal doxorubicin) (Figure 1A) and IL (irinotecan liposomes) (Figure 1B). By contrast, biotinylated isotype-matched control antibodies do not bind to either liposomal formulation, demonstrating the specificity of anti-PEG antibody binding. To investigate whether the presence of anti-PEG antibodies can cause hypersensitivity reactions to PLD, anti-PEG or control antibodies were intravenously injected into C57BL/6JNarl mice and allowed to circulate for 3 h before the mice were intravenously injected with 2 mg kg^{-1} PLD, and internal body temperature of the mice was monitored for another 2 h. Injection of 25 mg kg^{-1} anti-PEG IgG induced a large temperature drop (ΔT) in C57BL/6J mice, but injection of the same dose of anti-PEG IgM had no effect on internal body temperature (Figure 1C). Analysis of the areas above the temperature−time curves confirmed significant differences in hypothermia induced by anti-PEG IgG but not anti-PEG IgM as compared to isotype-matched control antibodies after systemic administration of PLD (Figure 1D). Hypothermia severity depended on the dose (Figure 1E) and time between anti-PEG IgG and PLD administrations (Figure 1F).

Examination of other mice strains revealed that BALB/c mice were refractory to injection of 25 mg kg^{-1} anti-PEG antibodies followed by injection of 2 mg kg^{-1} PLD, but NOD/SCID mice were at least 10-fold more sensitive than C57BL/6J mice. Injection of 2 mg kg^{-1} of two different anti-PEG IgG antibodies (3.3 and 6.3) before administration of PLD induced more severe hypothermia than injection of heat-aggregated mouse immunoglobulins, often used as a positive control for inducing hypersensitivity in mice (Figure 2A). NOD/SCID mice injected with a 5-fold higher dose of anti-PEG IgM

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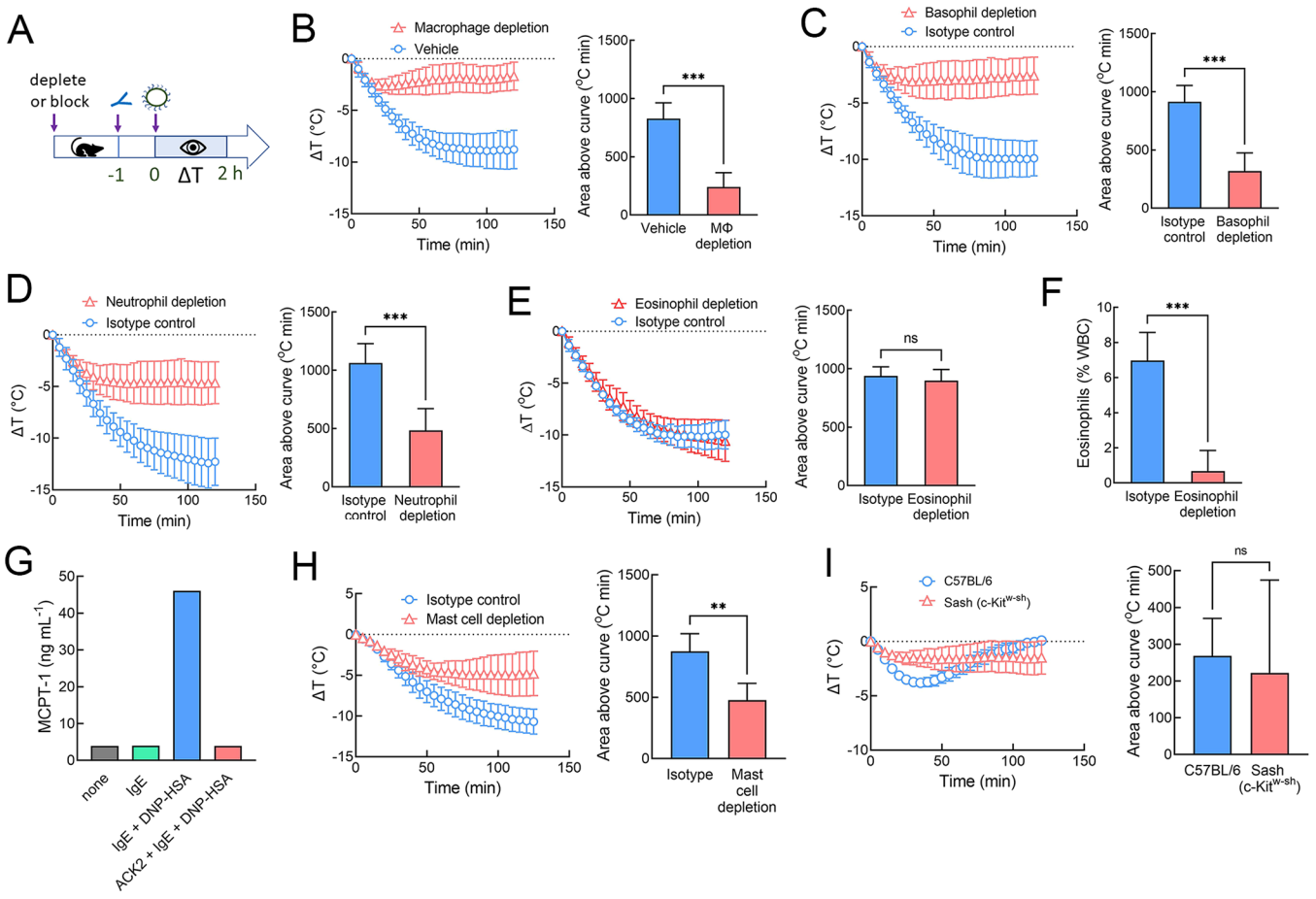

Figure 4. Depleting specific innate immune cells reduces HSRs against PLD in mice. (A) Schematic of approach used to test involvement of innate immune cells in HSR to PLD. NOD/SCID mice were pretreated with Clodrosome to deplete macrophages (B), Ba-103 antibody to deplete basophils (C), α-Ly6G antibody to deplete neutrophils (D), anti-mouse CCR3 antibody to deplete eosinophils (E), or ACK2 antibody to deplete mast cells (H) before injection of anti-PEG IgG and PLD. Vehicle or isotype-matched control antibodies were included in place of depleting or blocking agents in all experiments. Changes in body temperature (left) and mean values of the area above curves (right) are shown (Bars, SD; $n = 5$). (F) Effectiveness of eosinophil depletion by anti-mouse CCR3 antibody. (G) Effectiveness of mast cell depletion was assessed by measuring MCPT-1 in serum of untreated (none) mice or mice injected with anti-DNP IgE (IgE), DNP-HSA before anti-DNP IgE, or ACK2 anti-kit antibody to deplete mast cells before injection of DNP-HSA and anti-DNP IgE. (I) C57BL/6 or sash (c-Kit$^{w\text{-}sh}$) mice were intravenously injected with 25 mg kg^{-1} anti-PEG IgG 3 h before injection of 2 mg kg^{-1} PLD. Mean temperature drop (left) and area above the curves (right) are shown (Bars, SD; $n = 6$). Significant differences between mean values of depleted versus nondepleted mice are indicated: ns, not significant; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

(equivalent dose as IgG on a molar basis) experienced minor hypothermia that was not significantly greater than that observed in mice receiving 10 mg kg^{-1} control IgM (Figure 2A). Hypothermia depended on the dose of anti-PEG IgG with maximum effects observed at 2 mg kg^{-1} (Figure 2B). We also monitored other common symptoms of hypersensitivity such as altered lung function and reduced blood pressure. Both systolic and diastolic blood pressure of mice injected with anti-PEG IgG were significantly lower than those injected with isotype control antibody at 30 min postchallenge with PLD. (Figure 2C). Likewise, anti-PEG IgG significantly increased pause (Penh), an index of airway hyperresponsiveness, 30 min after PLD administration as compared to control IgG (Figure 2D). Mice injected with anti-PEG IgG and then treated with non-PEGylated LD generated significantly less hypothermia as compared to mice receiving PLD (Figure 2E), demonstrating dependence on the presence of PEG on the liposomes. Taken together, these results indicate that this simple model may help understand the mechanisms of PLD-induced hypersensitivity reactions.

Anti-PEG IgG Can Promote Binding of PEGylated Nanoparticles to Immune Cells via FcγII/III Receptors. To examine whether anti-PEG IgG can promote interactions between PEGylated nanoparticles and immune cells, we preincubated PEGylated quantum dots (QD655) with anti-PEG IgG antibodies (3.3 or 6.3) and then examined binding of the resulting mixtures to peripheral blood mononuclear cells isolated from NOD/SCID mice. Both anti-PEG IgG antibodies greatly increased binding of QD655 to peripheral blood mononuclear cells as compared to control IgG as shown by positive cell fluorescence at 655 nm (Figure 3A). Previous results showing that anti-PEG IgG antibodies but not anti-PEG IgM antibodies induce HSRs to PLD suggest a possible role for Fc gamma receptors (FcγR) in the response. FcγII (CD32) and FcγIII (CD16) are low-affinity receptors for IgG that are expressed on many types of immune cells.[42,43] Addition of mouse Fc Block, a rat anti-mouse monoclonal antibody against CD16/CD32, greatly decreased QD655 binding to PBMCs, demonstrating that anti-PEG IgG can bind to PEGylated nanoparticles and then bind to FcγII/III on blood cells (Figure

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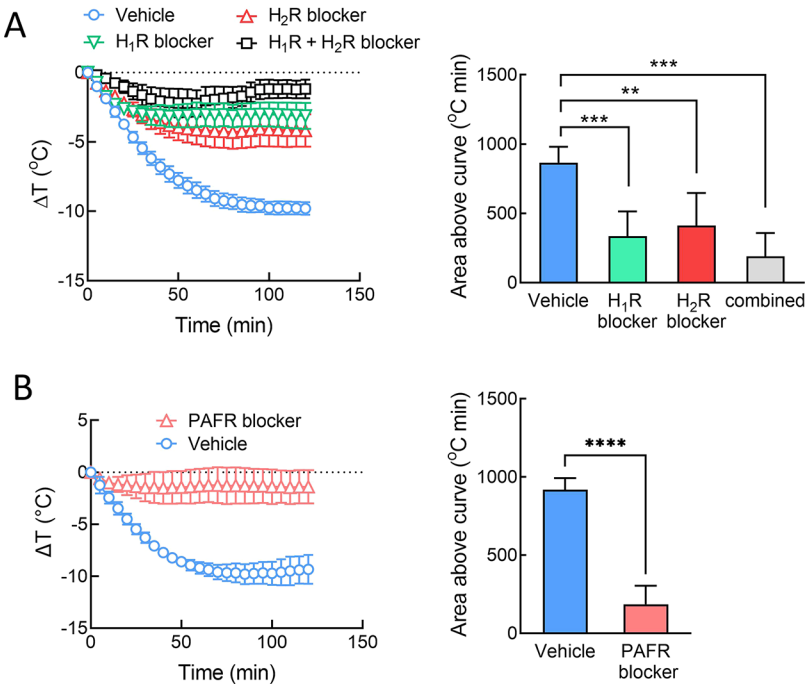

Figure 5. PAF and histamine are important mediators of anti-PEG IgG-induced hypersensitivity to PLD. (A) Mean temperature drop (left) and area above the curves (right) in NOD/SCID mice pretreated with histamine receptor 1 antagonist (cetirizine), histamine receptor 2 antagonist (ranitidine), or a combination of both before injection with 2 mg kg^{-1} anti-PEG IgG followed 1 h later with injection of 2 mg kg^{-1} PLD (Bars, SD; n = 5). (B) Mean temperature drop (left) and area above the curves (right) in NOD/SCID mice pretreated with PAF receptor antagonist (CV6209) before injection of 2 mg kg^{-1} anti-PEG IgG followed 1 h later with 2 mg kg^{-1} PLD (Bars, SD; n = 5). Significant differences between mean values are indicated: **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

3A). Nanoparticles are often taken up by phagocytes in the liver after intravenous administration. We therefore examined if anti-PEG IgG could promote specific interactions between QD655 and Kupffer cells (liver-resident macrophages) isolated from NOD/SCID mice. Anti-PEG IgG greatly increased the binding of QD655 to Kupffer cells as compared to cIgG (Figure 3B). QD binding to Kupffer cells was almost completed abrogated by addition of Fc Block, confirming interaction via FcγII/III receptors. FcγII/III receptors are also involved in HSR to PLD in NOD/SCID mice because blocking these receptors by intravenous injection of rat anti-mouse CD16/CD32 antibody significantly reduced hypothermia induced by PLD in the presence of anti-PEG IgG antibodies (Figure 3C).

Innate Immune Cells Are Involved in HSR Mediated by Anti-PEG IgG. FcγII/III receptors are expressed on a wide variety of innate immune cells. To identify possible immune cells involved in HSRs to PLD, we used well-established antibodies or drugs to selectively deplete specific populations of immune cells and then observe the degree of hypothermia induced by injection of anti-PEG IgG and PLD (Figure 4A). Depletion of macrophages with clodronate-containing liposomes significantly reduced hypothermia caused by administration of anti-PEG IgG and PLD, consistent with a role of liver macrophages in HSR (Figure 4B). Likewise, depletion of basophils (Figure 4C) or neutrophils (Figure 4D) significantly suppressed anti-PEG IgG induced hypothermia to PLD. By contrast, depletion of eosinophils did not affect the degree of hypothermia induced by anti-PEG IgG and PLD (Figure 4E), indicating that eosinophils do not play a role in hypersensitivity to anti-PEG IgG and PLD in NOD/SCID mice. Because we did not observe a strong effect for eosinophils, we confirmed that eosinophils were effectively depleted from the circulation of NOD/SCID mice after treatment with anti-mouse CCR3 antibody (Figure 4F). Mast cells also appeared to be involved in hypothermia induced by anti-PEG IgG and PLD. Pretreatment of mice with an antibody against CD117 (c-kit) expressed on the surface of mast cells resulted in functional depletion of mast cells as shown by loss of secretion of mouse mast cell protease (MCPT-1) when mice were challenged with DNP-BSA and anti-DNP IgE antibody (Figure 4G). Challenge of NOD/SCID mice with anti-PEG IgG and PLD after depletion of mast cells produced modest, but significant, hypothermia as compared to mice that were not treated with ACK2 antibody (Figure 4H). Less extreme hypothermia with different kinetics was induced by anti-PEG IgG and PLD in sash (c-Kit$^{w\text{-}sh}$) mice, which lack mast cells, as compared to C57BL/6 mice treated the same way, but there was not a significant difference in the area above the temperature curves (Figure 4I). These results indicate that mast cells play a minor role but macrophages, basophils, and neutrophils play major roles in anti-PEG IgG mediated hypothermia to PLD.

Anti-PEG IgG-Induced Hypersensitivity Is Mediated by PAF and Histamine. Platelet-activating factor (PAF) and histamine are important mediators of hypersensitivity reactions.[44] To examine if these mediators contribute to anti-PEG IgG-induced PLD hypersensitivity, NOD/SCID mice were pretreated with histamine receptor 1/2 or PAF receptor antagonists. Antagonism of either histamine receptor 1 or histamine receptor 2 significantly reduced hypothermia induced by anti-PEG IgG and PLD (Figure 5A). Combination of histamine receptor 1 and receptor 2 antagonists appeared to more completely block hypothermia, but the effect was not statistically significantly greater than single treatment with

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either receptor 1 or 2 antagonists (Figure 5A). Antagonism of PAF receptor signaling suppressed hypothermia at least as well as histamine receptor 1 or 2 antagonists (Figure 5B). These results show that both PAF and histamine are involved in anti-PEG IgG-induced hypersensitivity to PLD.

Anti-PEG IgG Can Induce Hypersensitivity to Diverse PEGylated Compounds. Doxorubicin, either as a free drug or encapsulated in liposomes, can cause immunogenic cell death (ICD) and might not be generally representative of other PEGylated nanomedicines.[45] We therefore tested if hypersensitivity reactions are induced in the presence of anti-PEG IgG by alternative PEGylated liposomes, nanoparticles, and proteins. We first examined IL, a liposomal formulation of the topoisomerase I prodrug irinotecan. The dose of IL was adjusted to achieve approximately equal PEG levels as PLD based on the lower PEG density (0.3 mol %) and larger size (~120 nm) of IL as compared to PLD (5 mol % PEG and ~90 nm diameter). Similar to results observed with PLD, NOD/SCID mice that were first iv injected with anti-PEG IgG displayed strong hypothermia after administration of IL (Figure 6A). We next examined smaller PEGylated solid Fe_3O_4 nanoparticles (30 nm diameter, modified with PEG_{2000}). The dose of the IONPs (6×10^{12}) was adjusted to approximately match the number of PLD. In common with PLD and IL, hypothermia was also observed in mice injected with 2 mg kg^{-1} anti-PEG IgG and 6×10^{12} IONPs (Figure 6B). Besides nanoparticles, we also examined whether PEGylated proteins can induce hypothermia in this model. Bovine serum albumin (BSA) was modified with an average of 13 PEG_{5000} chains to create a PEGylated protein (BSA-PEG_{13}). Mice injected with 2 mg kg^{-1} anti-PEG IgG and then approximately 6×10^{12} molecules of BSA-PEG_{13} displayed significantly more severe hypothermia as compared to injection of 6×10^{12} molecules of unmodified BSA (Figure 6C). In all instances, anti-PEG IgG produced significantly greater hypothermia as compared to cIgG. We conclude that an array of PEGylated compounds with widely different characteristics can induce hypothermia in mice in the presence of anti-PEG IgG.

Anti-PEG antibodies (3.3 or 6.3) bind to the repeating ethylene oxide subunits in the PEG backbone. However, antibodies that selectively bind to the methoxy functionality of mPEG are also generated when immunogenic PEGylated proteins are injected into animals.[46,47] Because nearly all PEGylated medicines use mPEG, we examined if anti-mPEG IgG (15-2b) can also induce hypersensitivity-like symptoms in mice. Injection of anti-mPEG IgG produced significantly greater hypothermia in mice as compared to a cIgG after administration of PLD (Figure 7A). The heavy chain of this anti-mPEG antibody (IgG_{2b}) differs from the heavy chain of the anti-PEG backbone (3.3 and 6.3) antibodies (IgG_1), which might affect binding to Fc receptors on innate immune cells. We therefore used CRISPR-Cas9 for *in vitro* class-switch recombination to change the heavy chain of 15-2b from IgG_{2b} to IgG_1. However, injection of 2 mg kg^{-1} anti-mPEG IgG_1 antibody (15-2c) before injection of PLD also caused severe hypothermia in mice (Figure 7B), indicating that this effect is not specific for mouse IgG_1 or IgG_{2b} antibodies. Recent reports suggest that some individuals develop IgE antibodies against PEG, which might cause anaphylactic reactions after administration of COVID-19 mRNA vaccines.[48,49] To examine if anti-PEG IgE can induce hypersensitivity reactions to a PEGylated nanoparticles, we switched the heavy chain of anti-PEG IgG (3.3) to IgE. Injection of between 0.5 and 2 mg kg^{-1} anti-PEG IgE before PLD administration produced obvious temperature drops in NOD/SCID mice as compared to control IgE (Figure 7C), showing that anti-PEG IgE antibodies can also induce hypersensitivity to PEGylated nanomedicines.

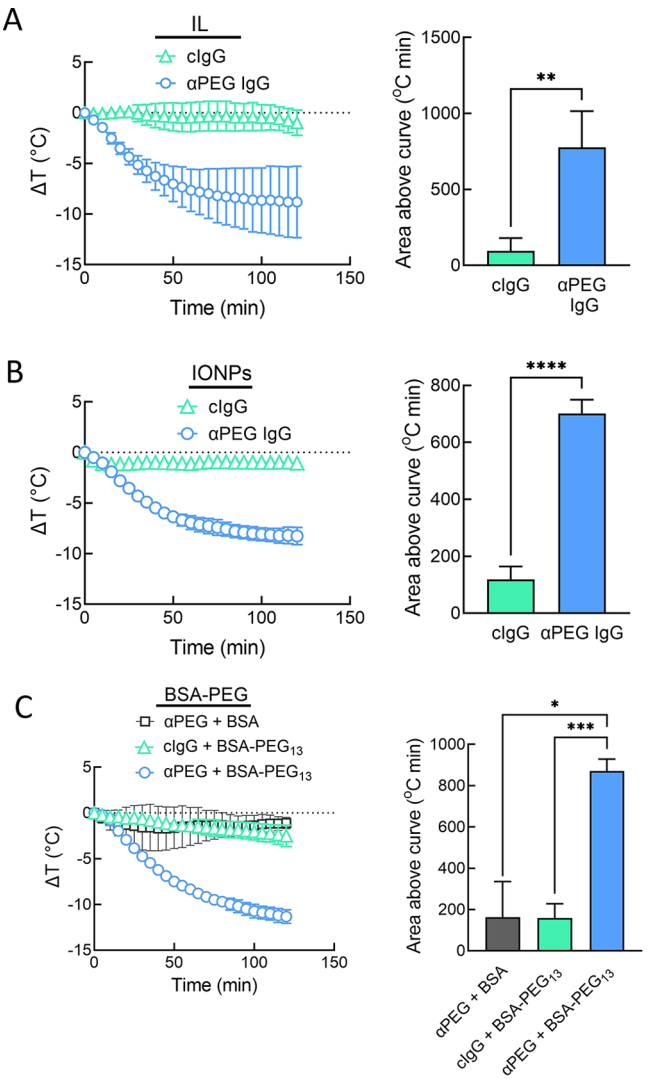

Figure 6. Other PEGylated agents can induce hypothermia in response to anti-PEG IgG. Mean temperature drop (left) and area above the curves (right) in NOD/SCID mice intravenously injected with 2 mg kg^{-1} anti-PEG IgG or cIgG 1 h before injection of (A) 12 mg kg^{-1} IL, (B) 6×10^{12} IONPs, or (C) 0.2 mg kg^{-1} BSA-PEG or 0.1 mg kg^{-1} BSA. (Bars, SD; $n = 5$). Significant differences between mean values are indicated: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$.

PEGylated Nanoparticles Can Interact and Activate Human Innate Immune Cells in the Presence of Human Anti-PEG Antibodies. We examined if human immune cells can also interact with and respond to PEGylated nanoparticles in the presence of anti-PEG antibodies. Humanized 3.3 (c3.3-IgG) and 6.3 (Hu-6.3) anti-PEG IgG antibodies were used for these experiments. c3.3-IgG is a chimeric antibody in which the constant region domains of the mouse 3.3 anti-PEG IgG antibody were replaced with the constant regions derived from human IgG_1. The antigen binding sites of this antibody are identical to murine 3.3 IgG. Hu-6.3, by contrast, is a humanized IgG in which the constant region genes as well as the variable chain framework regions of murine 6.3 anti-PEG

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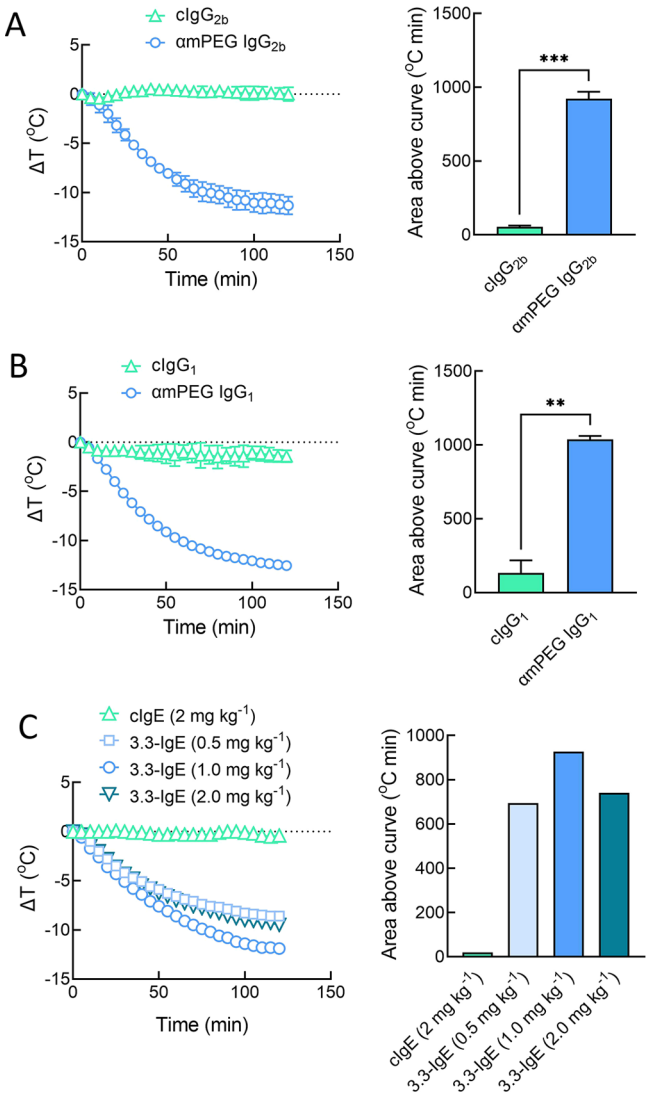

Figure 7. Anti-PEG antibodies with different specificity and heavy chains induce hypothermia in mice after injection of PLD. NOD/SCID mice were intravenously injected with (A) 2 mg kg^{-1} of anti-methoxy-PEG IgG$_{2b}$ (15-2b) or cIgG$_{2b}$ (n = 5), (B) 2 mg kg^{-1} of anti-methoxy-PEG IgG$_1$ (15-2c) or cIgG$_1$ (n = 5), or (C) the indicated doses of anti-PEG IgE (3.3-IgE) antibodies (n = 1) 1 h before injection of 2 mg kg^{-1} PLD. Mean temperature drop (left) and area above the curves (right) are shown (Bars, SD). Significant differences between mean values are indicated: **$p \leq 0.01$; ***$p \leq 0.001$.

IgG were replaced with the corresponding human IgG domains. PEGylated quantum dots (QD655) mixed with c3.3-IgG or Hu6.3 selectively bound to primary human basophils and neutrophils (Figure 8A). Binding to immune cells depended on anti-PEG IgG binding to the QDs because nonbinding human cIgG did not promote QD binding to either basophils or neutrophils. Similar results were observed for human monocytes that were allowed to attached to glass coverslips before human antibody/QD mixtures were added. QD fluorescence was clearly visible on monocytes challenged with human anti-PEG IgG and QD655 whereas no specific binding of QDs was observed with human cIgG plus QD655 (Figure 8B). Activation of human basophils by PLD in the presence of human anti-PEG IgG antibodies was also examined by measuring release of histamine. We used c3.3-IgG$_1$ and

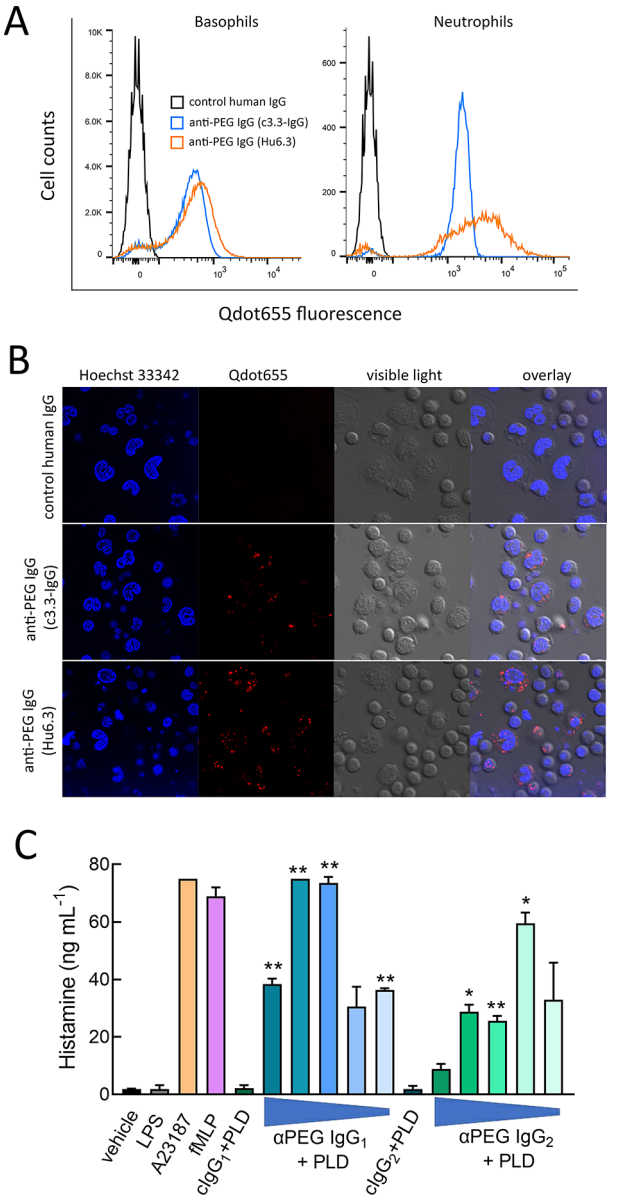

Figure 8. Interaction and activation of human immune cells by nanoparticles in the presence of human anti-PEG IgG. (A) Flow cytometric analysis of QD655 mixed with human cIgG or human anti-PEG IgG before addition to primary human basophils (left) or neutrophils (right). (B) QD655 mixed with human cIgG or human anti-PEG IgG were incubated with human peripheral blood mononuclear cells on glass slides. Cellular DNA was visualized with Hoechst 33342 (blue), whereas QD655 was visualized as red fluorescence on a confocal microscope. (C) Histamine concentrations in cultures of 3 × 10^4 primary human basophils incubated with the indicated control stimulators (vehicle, LPS, A23187, or fMLF) or 5-fold serial dilutions of PLD preincubated with a 50-fold molar ratio of human cIgG$_1$ or cIgG$_2$ or human anti-PEG IgG$_1$ or anti-PEG IgG$_2$ antibodies (n = 2). Significant differences between mean histamine concentrations with PLD + anti-PEG IgG vs PLD + cIgG are indicated: *$p \leq 0.05$; **$p \leq 0.01$.

c3.3-IgG$_2$, a chimeric antibody in which the mouse constant regions were replaced with constant regions derived from human IgG$_2$ because some PEGylated macromolecules are reported to primarily induce IgG$_2$ anti-PEG antibodies in patients and IgG$_2$ may predominate in pre-existing anti-PEG antibodies in normal donors.[16,50] Human basophils secreted

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histamine in response to the calcium ionophore A23187[51] as well as N-formylated tripeptide fMLF[52] but not to LPS[53] as expected (Figure 8C). PLD and anti-PEG IgG antibodies induced significant elaboration of histamine from basophils over a wide range of concentrations whereas PLD mixed with nonbinding human $cIgG_1$ or $cIgG_2$ did not induce significant release of histamine from basophils, indicating that human basophils can be activated by PLD coated with anti-PEG IgG antibodies (Figure 8C).

DISCUSSION

Hypersensitivity reactions (HSRs, also referred to as infusion reactions (IRs)) are complex, immune-mediated side effects that mainly occur within minutes to hours after receiving a therapeutic dose of intravenously administered drug product. These drug products are diverse and include a broad spectrum of low and high molecular weight molecules as well as nanomedicines. Clinically, HSRs occur in less than 5% of patients treated with such drug products, and severe life threatening events are rarer.[7] Among the HSRs to various drug products, those that result from exposure to PEGylated drug products are the most studied. To better understand the HSR mechanism of action, we investigated HSR to PEGylated liposomal doxorubicin (PLD) for which HSR has been studied in animals and humans. We examined if HSRs can be caused by interaction of PLD with antibodies that bind to poly(ethylene glycol) (PEG) in the corona of the PLD. We find that the presence of anti-PEG IgG but not anti-PEG IgM antibodies can induce symptoms of HSRs to PLD in mice, including hypothermia, reduced systolic and diastolic blood pressure, and altered lung function. More dramatic hypothermia is induced in NOD/SCID mice as compared to C57BL/6 and BALB/c mice, suggesting a major role for innate immune cells in the response. Studies using blocking or depleting agents revealed that hypothermia induced by PLD and anti-PEG IgG acts via FcγR II/III on macrophages, neutrophils, and basophils through secretion of histamine and platelet-activating factor (PAF). In addition, IgG antibodies recognizing the terminal methoxy functionality on mPEG as well as other PEGylated nanoparticles and a PEGylated protein could induce HSR in mice. We further demonstrated that humanized anti-PEG IgG antibodies bound to PEGylated nanoparticles can specifically interact with human immune cells and induce the release of histamine from human basophils. Taken together, our study describes an important role for anti-PEG IgG induced HSR via interaction of FcγRs on innate immune cells.

Accumulating evidence links the presence of anti-PEG antibodies to HSRs for a range of PEGylated medicines (Table 1). In many cases, HSRs are associated with the induction of anti-PEG antibodies by the administered PEGylated therapeutics. For example, PEGylated liposomes that encapsulate plasmid DNA induce the generation of anti-PEG IgG and severe HSR in mice.[54] Several PEGylated medicines using nonhuman proteins, such as porcine uricase, phenylalanine ammonia-lyase from cyanobacteria, or *E. coli* L-asparaginase induce anti-PEG antibodies that are associated with HSRs in patients.[19,20,55−58] On the other hand, HSRs in patients receiving pegnivacogin, a PEGylated aptamer, are believed to be caused by pre-existing anti-PEG antibodies present in the circulation of recipients.[28] Likewise, although PLD does not induce anti-PEG antibodies, infusion reactions against PLD are induced by anti-PEG antibodies in animal models.[59] Anti-PEG antibodies are present in many normal individuals that have never received PEGylated medicines,[15,16] consistent with a role for pre-existing anti-PEG antibodies in infusion reactions against PLD.

Table 1. Examples of Hypersensitivity Reactions Associated with Infusion of PEGylated Nanomedicines and Biomolecules[a]

substance	anti-PEG antibody status	host	citation
PEGylated liposome encapsulated oligonucleotides	induced	mice	60
PEGylated liposome encapsulated DNA	induced	mice	54
PLD	ND	humans	5, 6
PLD	pre-existing	pigs	59
PEGylated recombinant factor VIII	induced	hemophilia A patients	61
PEGylated recombinant porcine uricase	induced	gout patients	18, 55, 62
PEGylated cyanobacteria phenylalanine ammonia-lyase	induced	phenylketonuria patients	19, 56
PEGylated *E. coli* L-asparaginase	induced	leukemia patients	20, 57, 58
PEGylated *Erwinia* L-asparaginase	induced	ALL patients	63
PEGylated RNA aptamer	pre-existing	acute coronary syndrome patients	28, 29, 64, 65
PEGylated dimeric peptide	ND	anemia patients	66, 67

[a]Abbreviations: ND, not determined; ALL, acute lymphoblastic leukemia.

Our results identify a role for anti-PEG IgG antibodies acting through FcγRs in induction of HSRs against PEGylated nanomedicines and macromolecules. Fc receptors display selectivity for immunoglobulins with different heavy chain isotypes including receptors for IgA (FcαRI/CD89), IgM (FcμR), IgA/IgM (Fcα/μR), IgE (FcεRI), and IgG (FcγRI, FcγRII, and FcγRIII).[42] FcγRs, which bind to the glycosylated CH_2 domain of IgG molecules, are expressed on a variety of immune cells including monocytes, macrophages, neutrophils, basophils, eosinophils, NK cells, mast cells, dendritic cells, and B cells.[42,43] FcγRs preferentially interact with immunoglobulins that are bound to a surface such as a bacterium or viral particle as well as to immune complexes formed between multiple immunoglobulins and antigens, resulting in effector functions such as phagocytosis, degranulation, release of lipid mediators and cytokines, and antibody-dependent cellular cytotoxicity (ADCC) depending on the particular immune cell.[42,43] Allergen-specific IgG that forms immune complexes can bind to FcγRs on innate immune cells, resulting in cell activation and release of mediators that can induce infusion reactions.[68−70] Our results indicate that anti-PEG IgG bound to PLD can subsequently bind to FcγRII/III to activate innate immune cells including basophils, neutrophils, and macrophages, resulting in the release of histamine and platelet-activating factor. These soluble mediators can affect many organ systems to induce effects such as vasodilation, hypotension, bronchoconstriction, increased vascular permeability, abnormal heart rate, and cardiac contraction.[69]

Some nanomedicines and macromolecules such as dextran superparamagnetic iron oxide (SPIO) nanoparticles can induce HSRs even though they are not PEGylated.[71−73] Upon

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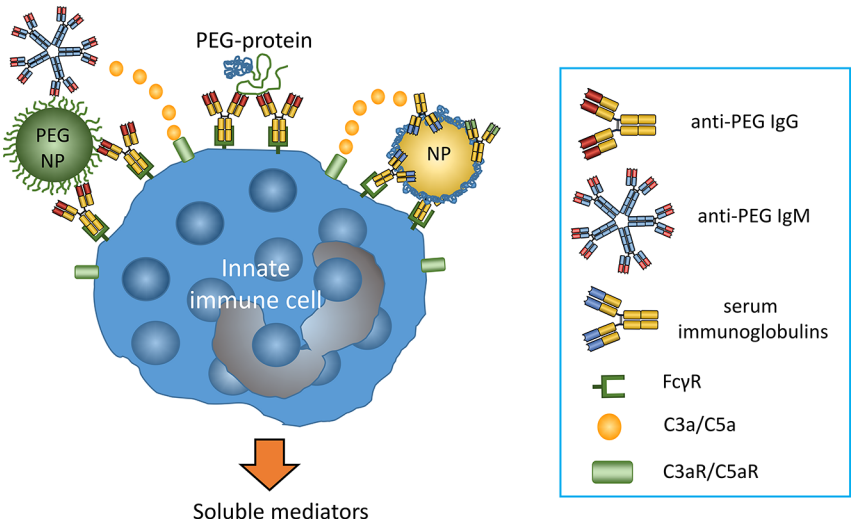

Figure 9. Schematic model of mechanism of infusion reactions to nanoparticles and PEGylated macromolecules. Anti-PEG IgG antibodies bound to PEGylated nanoparticles, liposomes, or macromolecules can cross-link FcγRs on innate immune cells to elaborate soluble mediators such as histamine and platelet activation factor to cause HSRs. Nonspecific IgG antibodies (serum immunoglobulins) may also bind to nanoparticles and cross-link FcγRs in an analogous fashion. IgG and IgM antibodies bound to nanoparticles may activate complement to produce anaphylatoxins (C3a and C5a) that may cooperate with FcγR signaling to further activate innate immune cells.

introduction to the human body, serum proteins rapidly bind and coat the nanoparticle surface to form a dynamic corona.[74] The corona includes common serum proteins including IgG which is found at high concentrations in serum.[75] IgG antibodies present in the protein corona can activate complement on a variety of liposomes and nanoparticles.[76] We speculate that serum immunoglobulins present in the NP protein corona may act like anti-PEG antibodies to cluster FcγRs on innate immune cells to induce HSRs in some patients (Figure 9). This also suggests that creating targeted nanomedicines with antibody fragments lacking the Fc domain may reduce possible risks of inducing HSRs by immobilized whole antibodies.

Complement activation by anti-PEG IgM antibodies is associated with HSRs to PEGylated liposomes and nanoparticles in a process termed complement activation-related pseudoallergy (CARPA).[6,10] Anti-PEG antibodies that bind to PEG on PEGylated nanoparticles activate the complement cascade, which liberates complement products with diverse biological roles. The reaction products C3b and iC3b can physically attach to the protein corona on nanoparticles to enhance phagocytosis and accelerate the rate of nanoparticle clearance.[77,78] The terminal complement reaction product termed the membrane attack complex can destabilize the structural integrity of PEGylated liposomes and nanoparticles to cause premature release of molecular cargos.[25,27] The anaphylatoxins C3a and C5a can bind and activate innate immune cells that express C3aR1 and C5aR1 receptors.[79] Activation of complement by anti-PEG IgM antibodies produces strong hypersensitivity reactions in pigs immediately after infusion of PEGylated liposomes.[59] The significance of anti-PEG mediated complement activation in HSRs to PEGylated nanomedicines remains unclear due to differences in complement-mediated hypersensitivity reactions in humans versus pigs.[3,7,13] We did not observe hypothermia or other signs of HSR in our mouse models with PLD in the presence of anti-PEG IgM at the same mass or molar doses as anti-PEG IgG, even though anti-PEG IgM is an effective activator of complement on PLD.[25] However, mice have much lower complement activity than humans and many other experimentally used animals including rats, rabbits, and guinea pigs.[3,80] In the present study, a human anti-PEG IgG antibody specifically accumulated PEGylated nanoparticles on human basophils, neutrophils, and macrophages and activated human basophils to secrete histamine, a well-known effector of hypersensitivity reactions, in the presence of PLD, consistent with an important role for anti-PEG IgG in HSRs in human patients. We speculate that complement reaction products generated by anti-PEG IgG and IgM antibodies as well as serum immunoglobulins (antibodies) present in the protein corona surrounding nanoparticles may act cooperatively with anti-PEG IgG bound to PEGylated nanoparticles, liposomes, and macromolecules to activate innate immune cells and initiate HSRs (Figure 9).[13,81] Additional studies to determine the relative contributions and roles of anti-PEG IgM and IgG antibodies to HSRs against PEGylated medicines in human patients are clearly warranted.

Besides PLD, other NPs including IL and PEGylated iron oxide NPs as well as a PEGylated protein also induced symptoms of HSR in the presence of anti-PEG IgG. IL is a liposomal form of irinotecan which is larger than PLD (~120 nm vs ~90 nm) and displays lower levels of PEG_{2000} on the surface (0.3 mol % vs 5.3 mol %) as compared to PLD. The iron NPs are ~30 nm in diameter and are coated with an unknown amount of PEG_{2000}. Bovine serum albumin has a molecular weight of about 66,000 Da and was modified with an average of 13 PEG_{5000} molecules. We also observed HSR with both anti-mPEG and anti-PEG antibodies. Both 3.3 and 6.3 anti-PEG antibodies bind to approximately 12−16 repeating ethylene oxide subunits present in the backbone of PEG,[82,83] whereas the anti-mPEG antibody (15-2b) binds to the terminal methoxy moiety as well as approximately seven ethylene oxide subunits.[84] Thus, HSR is induced by antibodies that bind different portions of PEG in the presence of a variety of PEGylated nanoparticles and biomolecules with a range of physical properties and PEG sizes, indicating that this phenomenon is general.

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A severe form of HSR called anaphylaxis is induced when allergens cross-link immunoglobulin E (IgE) antibodies bound by Fc epsilon receptors (FcεRI) on mast cells or basophils, which then rapidly release histamine. Anti-PEG IgE antibodies have been implicated in some HSRs to PEG.[48,85,86] IgE antibodies against PEG have also been implicated in rare anaphylactic reactions to PEGylated liposomal contrast agents and COVID-19 mRNA vaccines.[87,88] However, no studies have directly demonstrated that anti-PEG IgE antibodies can induce HSRs to PEGylated nanoparticles or mRNA vaccines. We used in situ class-switch recombination of the immunoglobulin heavy chain gene to create a mouse IgE antibody that displays the same variable region as the anti-PEG IgG used in our study. Our results show that anti-PEG IgE can induce symptoms of HSR in mice after administration of PLD, indicating that anti-PEG IgE is potentially important in some hypersensitivity reactions to PEGylated compounds. Our model may help shed more light on the role of anti-PEG IgE in anaphylactic reactions to PEG.

Some recent studies demonstrated a role for anti-PEG IgG mediated activation of basophils in allergic responses to mRNA COVID-19 vaccines.[89,90] This is consistent with the results of our study showing that anti-PEG IgG can bind to PEGylated nanoparticles and cross-linking Fcγ receptors on basophils. It is noteworthy that three individuals with known PEG allergy displayed dose-dependent basophil activation when challenged with BNT162b2 as well as with PEGylated liposomal doxorubicin but not with free (unconjugated) PEG.[90] PEG is stably integrated in the lipid bilayer of Doxil as a PEG_{2000} lipid conjugate with 18 carbon lipid tails and is semistably anchored on the surface as PEG_{2000} attached to neutral lipids with lipid tails containing 14 carbons in the BNT162b (Comirnaty) vaccine from BioNTech, Pfizer, and Fosun Pharmaceutical and mRNA-1273 vaccine from Moderna.[30,91] Anti-PEG antibodies display much stronger avidity for PEG molecules bound to a surface (such as a nanoparticle or liposome) or conjugated to a macromolecule (such as a protein or lipid molecule) as compared to unconjugated PEG.[30] Anti-PEG antibody binding to free PEG is also highly dependent on PEG size, with a million-fold difference in binding avidity found for PEG_{5000} as compared to PEG_{35000}.[30] The use of high molecular weight PEG or PEGylated nanoparticles might improve the sensitivity of skin tests to detect PEG sensitivity in patients.

Rodent models are considered to be relatively insensitive to HSR caused by liposomal pharmaceuticals and nanoparticles.[92] We found, however, that NOD/SCID mice were relatively sensitive models as compared to other common laboratory strains (BALB/c and C57BL/6). The increased sensitivity may be related to lack of competition for FcγR binding by endogenous immunoglobulins since NOD/SCID mice lack functional T and B cells.[93] Although NOD/SCID mice were used for most studies, consistent results were observed in C57BL/6 mice. In addition, humanized anti-PEG IgG antibodies bound to PEGylated nanoparticles selectively associated with innate immune cells from normal human donors and induced the activation of human basophils in the presence of PLD.

We preinjected anti-PEG monoclonal antibodies before administration of PEGylated liposome, nanoparticles, or proteins. This contrasts with preimmunization of mice with a PEGylated protein to generate polyclonal anti-PEG antibodies before challenge with a PEGylated therapeutic. We previously reported that both methods caused similar accelerated blood clearance, altered biodistribution, and decreased anticancer activity of PLD in a tumor-bearing mouse model,[22] indicating that both approaches are useful to study the effects of anti-PEG antibodies on PEGylated medicines. Injection of monoclonal anti-PEG antibodies provides some advantages including the ability to differentiate the effects of different antibody classes (ie., IgG, IgM, IgE) and specificities (anti-PEG versus anti-mPEG) as well as to more precisely control anti-PEG antibody serum concentrations.

Induction of HSRs to PEGylated nanoparticles and biomolecules likely depends on the stoichiometry between antibodies and nanoparticles. Between three high-affinity and 15 low affinity anti-PEG antibodies per PEGylated entity cause formation of immune complexes associated with accelerated blood clearance and reduction in biological activity.[22,23,94,95] Likewise, multiple anti-PEG antibodies per PLD are required to activate the complement cascade and destabilize liposome integrity.[25] Most normal individuals have moderate levels of anti-PEG IgG in their circulation, but a few percent have high levels of pre-existing anti-PEG IgG (>10 $\mu g\ mL^{-1}$), in the range of anti-PEG IgG concentrations that exceed the threshold that may induce HSRs.[30] In our study, we observed strong HSR symptoms when mice were injected with 2 mg kg^{-1} anti-PEG IgG in the presence of PLD, which corresponds to an anti-PEG IgG serum concentration of approximately 25 $\mu g\ mL^{-1}$.[96] We note that 1.0% of Han Chinese in Taiwan never exposed to PEGylated medicines have pre-exiting anti-PEG IgG antibodies in their circulation exceeding this concentration.[15,30] The sensitivity to anti-PEG IgG in mice and humans likely differs, but this indicates that the anti-PEG antibody concentration used in our study is reasonable. In addition, the incidence and concentrations of anti-PEG antibodies in the general population may be increasing due to the widespread administration of the BNT162b and mRNA-1273 SARS-CoV-2 vaccines. These vaccines contain nucleoside-modified mRNA in lipid nanoparticles with PEG_{2000} attached to their surface. A recent study found that the titer of pre-existing anti-PEG IgG was significantly boosted by an average of 13.1-fold following mRNA-1273 vaccination and 1.78-fold after BNT162b2 vaccination.[97] Anti-PEG IgM concentrations increased even more with levels rising an average of 68.5-fold for mRNA-1273 and 2.64-fold following BNT162b2 vaccination.[97] The increased levels of anti-PEG antibodies may substantially increase the population of individuals at risk of developing HSRs against PEGylated nanopharmaceuticals and macromolecules.

CONCLUSIONS

Anti-PEG IgG antibodies can induce hypersensitivity reactions after binding to PEG on PEGylated nanoparticles, liposomes, or macromolecules by interacting with FcγRs on basophils, neutrophils, and macrophages to induce the release of PAF and histamine. More generally, we speculate that immunoglobulins in the protein corona on the surface of nanoparticles may contribute to HSRs by a similar mechanism. Increased understanding of the pathways leading to adverse effects of nanomedicines and macromolecules should allow rational design of assays to prescreen patients for safer and more effective therapy as well as create medicines that evade induction of hypersensitivity reactions in patients.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.2c12193.

Sources of antibodies used in the study (PDF)

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Author Contributions

$^{\nabla}$W.-A.C. and D.-Y.C. made equal contributions.

Notes

The authors declare the following competing financial interest(s): Yechezekel Barenholz is one of the inventors on two already expired (March 2010) patents relevant to Doxil: (1) Barenholz, Y.; Haran, G. Method of Amphipathic Drug Loading in Liposomes by pH Gradient. U.S. Patent 5,192,549, March 9, 1993; U.S. Patent 5,244,574, September 14, 1993. (2) Barenholz, Y.; Haran, G. Liposomes: Efficient Loading and Controlled Release of Amphipathic Molecules. U.S. Patent 5,316,771, May 31, 1994. The Hebrew University received royalties from Doxil sales until the patents expired. Yechezekel Barenholz is also the CEO of Ayana Pharma LTD that developed and commercialized a Doxorubicin HCL liposomal injection (generic Doxil) that was approved by US FDA on October 12, 2021. B. M. Chen 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). The other authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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