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Polyethyleneimine is a potent systemic adjuvant for glycoprotein antigens

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Abstract

Polyethyleneimine (PEI) is an organic polycation used extensively as a gene and DNA vaccine delivery reagent. Although the DNA targeting activity of PEI is well documented, its immune activating activity is not. We recently reported that PEI has robust mucosal adjuvanticity when administered intranasally with glycoprotein antigens. Here, we show that PEI has strong immune activating activity after systemic delivery. PEI administered subcutaneously with viral glycoprotein (HIV-1 gp140) enhanced antigen-specific serum IgG production in the context of mixed T_h1/T_h2-type immunity. PEI elicited higher titers of both antigen binding and neutralizing antibodies than alum in mice and rabbits and induced an increased proportion of antibodies reactive with native antigen. In an intraperitoneal model, PEI recruited neutrophils followed by monocytes to the site of administration and enhanced antigen uptake by antigen-presenting cells. The T_h bias was modulated by PEI activation of the Nlrp3 inflammasome; however its global adjuvanticity was unchanged in Nlrp3-deficient mice. When coformulated with CpG oligodeoxynucleotides, PEI adjuvant potency was synergistically increased and biased toward a T_h1-type immune profile. Taken together, these data support the use of PEI as a versatile systemic adjuvant platform with particular utility for induction of secondary structure-reactive antibodies against glycoprotein antigens.

Keywords: adaptive immunity, innate immunity, PEI, vaccine, virus

Introduction

The field of vaccine adjuvants has expanded rapidly over the past decades following the discovery of innate immune receptors such as the Toll-like receptors, Nod-like receptors and Rig-like receptors (TLRs, NLRs and RLRs, respectively) responsible for activating and conditioning innate and adaptive immunity (1, 2). The requirement to demonstrate the necessity and safety of novel adjuvants has hampered their acceptance, resulting in limited licensing of vaccines containing novel adjuvants. New vaccines, particularly those needing to elicit high titer antibody responses against weakly immunogenic antigens such as HIV-1 gp140, may require more potent and/or tailored adjuvants. Adjuvant discovery therefore remains an important area of vaccine research.

We recently reported that the organic polycation polyethyleneimine (PEI) adjuvants viral glycoprotein antigens via the mucosal route, activating robust and protective immunity against influenza and herpes simplex virus-2 after a single intranasal administration of the relevant antigen co-formulated with PEI (3). The mechanism of innate immune activation was dependent upon the Irf3 IFN response pathway triggered by activation of intracellular dsDNA sensors. Additionally, PEI reversibly sequestered antigen, recruited antigen-presenting cells (APC) to the site of administration and targeted antigen to APC (3). Given these properties, we hypothesized that PEI might also have systemic immune stimulating activity. To investigate this, we tested PEI in both subcutaneous and intraperitoneal models of immunization. Here, we show that PEI elicits robust systemic antigen-specific antibody responses associated with a mixed T-helper cell response, which can be further enhanced and redirected toward a T_h1

bias by using PEI to co-deliver the TLR9 agonist CpG oligodesoxynucleotide (ODN). PEI therefore shows promise for use alone or as a platform system in combination with nucleic acid TLR agonists, as a systemic adjuvant.

Methods

Antigens and adjuvants

HIV-1 $gp140_{97CN54}$ was manufactured to GMP specification by Polymun Scientific (Vienna, Austria). Alexa-467-conjugated ovalbumin (OVA-Alexa-647) was purchased from Molecular Probes. The following adjuvants were used: PEI (linear 40 kDa and 160 kDa from Polymer Chemistry Innovations Inc.; branched 25 kD and 750 kDa from Sigma); aluminium salts (Imject alum; Thermo Scientific); Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) from Sigma; immunostimulatory CpG ODN 1826 (MWG Biotech) or CpG ODN 2395 (MWG Biotech).

Animals and immunizations

BALB/c mice were bred at the Sir William Dunn School of Pathology, Oxford University. C57BL/6 and C57BL/6.Nlrp3$^{-/-}$ mice were bred at the Department of Immunobiology, Yale University. C57BL/6.Irf3$^{-/-}$ mice were bred at the Division of Microbiology, Immunology and Glycobiology, Lund University. All animal experiments were performed under the appropriate national licenses in accordance with the UK Animals (Scientific Procedures) Act 1986 and were authorized by the UK Home Office and the Oxford local institutional ethical review board.

Model antigens and adjuvants were premixed and incubated for 2 h at 4°C prior to injection (PEI, alum) or formulated according to the manufacturer's instructions (FCA/FIA). Experimental immunogen formulations were prepared under sterile conditions in endotoxin-free PBS and were administered via the subcutaneous (100 µl) or intraperitoneal (500 µl) route. Adjuvant doses were based on typical experimental doses published earlier (alum, FCA/FIA) or determined in pilot studies using dose ranges of PEI. For subcutaneous immunization, 80–100 µg of PEI were chosen as the optimal dose range in mice, since this amount typically induced a robust adjuvant effect while causing only a moderate weight loss on day 1 after immunization as a measure of adjuvant toxicity/reactogenicity. PEI-induced weight loss was lower or statistically indistinguishable from the weight loss caused by standard experimental doses [1 mg $Al(OH)_3$] of alum (data not shown).

NZW rabbits (n = 4) were primed with 50 µg HIV-1 gp140 antigen mixed with 202.52 µg PEI, antigen mixed with Imject® Alum (1:1 v/v; Thermo Scientific) or antigen emulsified in FCA (1:1 v/v). At week 3, rabbits received a boost immunization with 20 µg gp140 in equivalent formulations (FCA was replaced by FIA). At week 6, 9 and 18, rabbits received boost immunizations with 20 µg gp140 antigen without adjuvant.

ELISA

Serum samples were cleared by centrifugation and stored at −20°C until analyzed. Antigen-coated and blocked ELISA plates were incubated with serial dilutions of samples. Bound antibodies were detected with the appropriate secondary reagents (anti-mouse IgG-HRP; STAR120P; Serotec); anti-mouse IgG1-HRP, IgG2a-HRP (BD Biosciences) and IgG2c-HRP (gtx77297; Genetex); anti-rabbit IgG-HRP (Sigma-Aldrich) and TMB substrate (Thermo Fisher Scientific) using previously described methods (3).

For the detection of antibodies reactive with denatured antigen, 16 µg antigen were denatured with 70 mM SDS and 100 mM dithiothreitol in a total volume of 50 µl PBS for 5 min at 95°C and transferred to 16 ml PBS for coating of high-binding ELISA plates overnight at 4°C.

Neutralization assays

The neutralization capacity of rabbit immune sera was assessed in a HIV-1 pseudovirus neutralization assay described previously (4). Briefly, serial dilutions of heat-inactivated immune sera were preincubated with pseudovirus for 1 h before addition of TZM-BL target cells. The infection level was determined after 24 h by measuring luminescence of cell lysates.

T-cell assays

For gp140-specific responses, splenocytes were cultured in the presence or absence of 10 µg/ml antigen for 48 h. Supernatants were cleared by centrifugation and analyzed by multiplex cytokine assay.

Cell recruitment assays

Mice were euthanized 4 or 24 h after immunization and small volume (2 ml) and large volume (5 ml) peritoneal lavages sequentially performed using ice-cold PBS-EDTA. Supernatants from small volume lavages were used in cytokine/chemokine analyses, cells from both lavages were combined for flow cytometry.

Multiplex cytokine assays

Supernatants were separated from either peritoneal lavage or cultured cells via centrifugation and stored at −80°C until use. Cytokine concentrations of neat cell culture supernatants or peritoneal lavage fluid were determined via Bio-Plex® cytokine array (Bio-Rad Laboratories).

Flow cytometry and antibodies

Peritoneal leucocytes were stained for flow cytometry and absolute numbers of B cells (CD11b$^-$ CD19$^+$), T cells (CD11b$^-$ CD3$^+$), monocytes (CD11b$^+$ Ly6C^{++} Ly6G$^-$ F4/80^{int}), macrophages (CD11b$^+$ F4/80^{hi} Ly6G$^-$ Ly6C$^-$), neutrophils (CD11b$^+$ Ly6G^{hi} Ly6C$^+$ F4/80$^-$), eosinophils (CD11b$^+$ Ly6C^{lo} Ly6G^{int} F4/80^{lo} SSC^{hi}) and dendritic cells (DC; CD11b$^{-/int}$ CD11c^{hi} F4/80$^{-/lo}$ MHC-II^{hi}) were determined.

The following antibodies were used for flow cytometry: CD11b (M1/70), CD19 (ID3), CD3 (145-2C11), Ly6C (AL-21), Ly6G (1A8), F4/80 (CI:A3-1), CD11c (HL3), MHC-II (2G9).

ELISpot

To detect gp140-specific cell frequencies, we used two overlapping 15-mer peptide pools spanning the N-terminal

(C1-C3) or C-terminal (C3-MPER) region of HIV-1 gp140$_{96ZM651}$. Briefly, 2×10^5 splenocytes per well were incubated on anti-IFN-γ-coated ELISpot plates at 37°C for 16 h in the presence of the peptide pools (2.5 μg/ml) in triplicate. IFN-γ production by gp140-specific T cells was quantified by counting spot-forming cells using an automated ELISpot reader. The threshold was determined as the mean background response plus 1 SD.

Statistical analysis

Titer data were $\log_{10}$-transformed and then tested for normality (Kolmogorov–Smirnov test). If data showed a normal distribution within each compared group, a one-way analysis of variance (ANOVA) was used to assess for statistical significance defined as $P < 0.05$. If the data were not normally distributed, they were analyzed using a Kruskal–Wallis test with the same significance limit. Direct comparisons between individual groups were tested for significance using the appropriate post-tests as indicated.

Results

Systemic adjuvant activity of different forms of PEI

PEI exists in different molecular forms including branched and linear, high and low molecular weight (5–7). To determine whether different forms of PEI show systemic adjuvant activity in combination with a viral glycoprotein model antigen (endotoxin-free HIV-1$_{97CN54}$ gp140), we immunized mice subcutaneously with gp140 coformulated with linear or branched PEI with molecular weights ranging from 25 to 750 kDa (Fig. 1a). The maximal adjuvant effect was observed after two immunizations, with up to 314-fold increased geometric mean endpoint titers per group compared to the control group receiving gp140 alone (no adjuvant, Fig. 1b). Antigen-specific endpoint titers plateaued after the final immunization and remained stable for 15 weeks, with significantly increased endpoint titers for all adjuvanted groups at week 24 (Fig. 1c). To interrogate the immune bias induced by different forms of PEI, we determined antigen-specific IgG1 and IgG2a titers as indicators of T_h2- or T_h1-biased immune responses, respectively. In PEI-immunized animals, induction of antigen-specific IgG2a was particularly pronounced compared to the control group, resulting in a significantly lowered IgG1/IgG2a titer ratio (Fig. 1d). These data demonstrate that all tested forms of PEI show a quantitatively and qualitatively equivalent adjuvant effect that is characterized by a moderate T_h1 bias compared to antigen alone.

A side-by-side comparison of PEI with highly potent systemic Freund's Adjuvant, consisting of priming with FCA and boosting with FIA, and alum in mice showed significantly improved performance of PEI compared to alum (Fig. 2a and b). The PEI-adjuvanted immune response was characterized by an intermediate IgG1/IgG2a endpoint titer ratio for PEI between that of FCA/FIA (T_h1) and alum (T_h2) adjuvants (Fig. 2c), indicating a mixed T_h1/T_h2 immune response. This was confirmed by the cytokines released from antigen-restimulated splenocytes of mice immunized with gp140 and PEI, which produced significant amounts of T_h1 cytokines IL-2, TNF-α and GM-CSF and the T_h2-associated cytokine IL-5 (Fig. 2d).

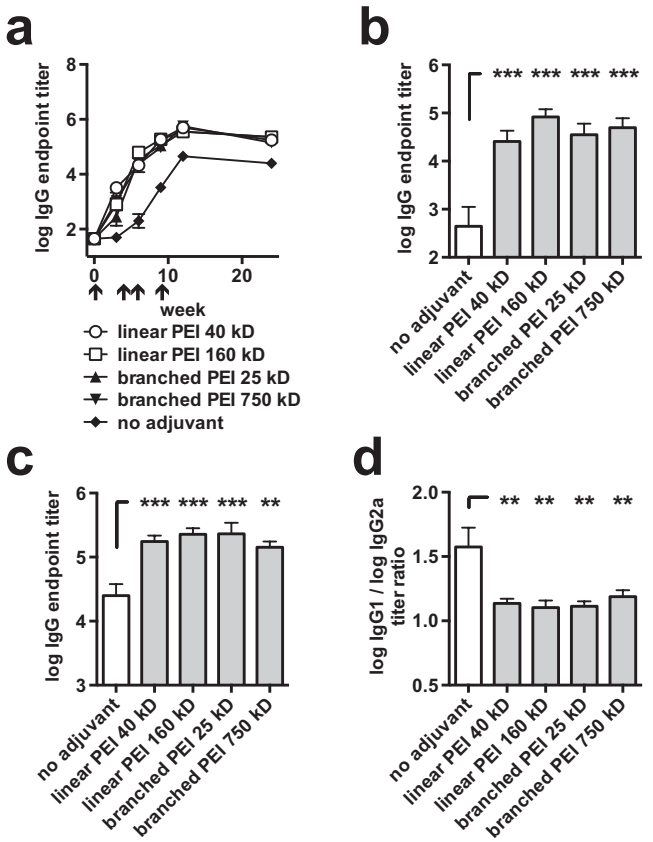

Fig. 1. Systemic adjuvant activity of different forms of PEI. (a–d) BALB/c mice (n = 6/group) were subcutaneously immunized four times (↑) with 1 μg gp140 alone or combined with 80 μg PEI [linear or branched, relative molecular weight (kDa) as indicated]. Bleeds were collected at 3 weeks post-prime and in week 6, 9, 12 and 24. (a) Time course of gp140-specific serum pan IgG endpoint titers. (b–c) Week 6 gp140-specific serum pan IgG (b) and week 24 pan IgG (c) endpoint titers. (d) Week 24 log IgG1/log IgG2a titer ratio. Log_{10}-transformed titer data were analyzed by ANOVA followed by Dunnett's post-test. Data are presented as mean ($\log_{10}$-transformed data) ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

PEI preferentially induces antibodies against native antigenic structures in rabbits

To confirm the adjuvant activity of PEI in a second, non-rodent species, we immunized rabbits subcutaneously using gp140 coformulated with PEI, FCA/FIA or alum, using a 50-fold lower dose (w/v) of PEI compared to alum. Rabbits received two subcutaneous immunizations of antigen with adjuvant, followed by three subcutaneous immunizations with the vaccine antigen alone. This extended immunization schedule was chosen to allow an optimal affinity maturation of the antibody response against the native antigen and to allow the generation of potent HIV-1 neutralizing antibodies, which was demonstrated to require several steps of B-cell affinity maturation (8). Similar to the data obtained in mice (Fig. 2a and b), PEI showed a trend toward increased adjuvant potency compared to alum (Fig. 3a and b) but due to small group sizes (n = 4), this effect did not reach significance. Since most broadly neutralizing antibody epitopes on HIV-1 Env are conformation dependent (9–11), we tested the ability of antigen-specific

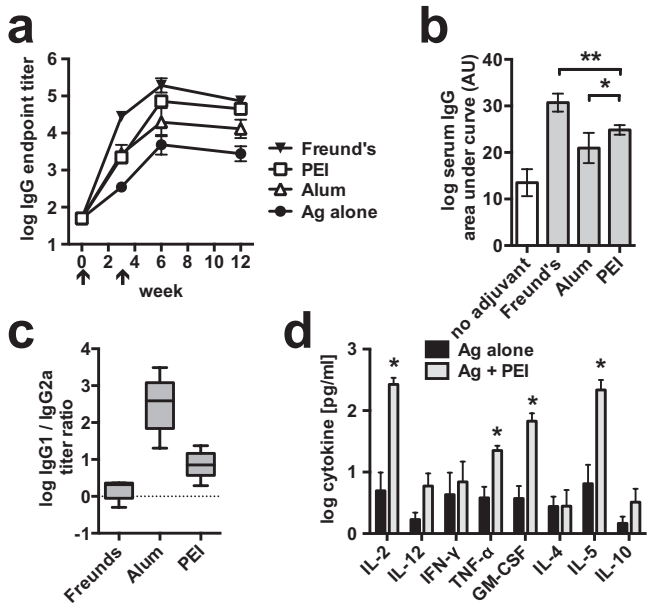

Fig. 2. Subcutaneous adjuvant activity of 25 kDa PEI compared to FCA/FIA and alum and cellular cytokine response. (a–c) BALB/c mice (n = 5/group) were subcutaneously immunized twice (↑) with 5 μg of gp140 alone or combined with FCA/FIA, 2 mg aluminium salts (alum) or 90 μg PEI at weeks 0 and 3. (a) Time course of gp140-specific serum pan IgG endpoint titers. (b) Week 0–12 area under serum pan IgG time course curve as a measure of the total immunogenicity of the formulations. (c) Week 12 log IgG1/log IgG2a serum titer ratio. (d) In a separate experiment, mice (BALB/c n = 5/group) were subcutaneously immunized with 50 μg of gp140 with or without 90 μg PEI at weeks 0 and 3 and the splenocytes restimulated with gp140 for 48 h 7 days later. Cytokine expression was assessed in the supernatant by T_h1/T_h2 Bio-Plex multiplex assay. Area under curve data were analyzed by ANOVA with Bonferroni correction. Cytokine secretion measurements were $\log_{10}$-transformed and analyzed by multiple t-tests (one per cytokine) adjusted with the Holm-Sidak correction. Data are presented as mean $\log_{10}$-transformed ± SEM. AU, arbitrary unit; $^*P < 0.05$; $^{**}P < 0.01$.

antibodies elicited by PEI-adjuvanted antigen to bind native compared to denatured gp140. Sera from rabbits immunized with PEI showed increased native/denatured antigen binding titer ratios compared to animals immunized with FCA/FIA and alum (Fig. 3c). This indicates better conservation and/or presentation to B cells of the antigenic secondary structure by PEI compared to the other adjuvants. Consistent with this finding, we observed a similar *in vitro* neutralization capacity of immune sera from FCA/FIA and PEI-adjuvanted animals against HIV-1 pseudoviruses, despite the fact that immune sera from the FCA/FIA group contain higher endpoint titers of HIV-1 gp140-reactive antibody by ELISA.

PEI induces cytokine secretion and leucocyte recruitment at the site of administration and targets antigen to APC

To characterize early events in the immune response to PEI administration, we used an established mouse model of acute inflammation, the peritonitis model (12). Mice were administered 70 μg PEI intraperitoneally and the cytokine/chemokine profile assayed in peritoneal lavage 4 or 24 h later (Fig. 4a). An acute inflammatory (IL-1β) response was evident, with characteristics of both T_h2-biased (IL-5, IL-13, G-CSF) and T_h1-biased (IFN-γ, IL-6, MIP-1α, KC) profiles. Concomitant with the cytokine release, intraperitoneal administration of PEI resulted in significant recruitment of immune cells into the peritoneal cavity. Cells recovered by lavage at 4 and 24 h were double-labeled for leukocyte markers and analyzed by flow cytometry. Fig. 4b shows cell counts in the peritoneal exudate in mice administered PEI compared to PBS. Most striking are the 4 h neutrophil and eosinophil infiltrates that decline to near baseline by 24 h and the increase in monocytes at 4 and 24 h. Resident peritoneal B cells migrate out of the peritoneum upon PEI stimulation, but not after non-inflammatory PBS injection. To investigate which cells acquired antigen, mice were injected intraperitoneally with fluorescently labeled OVA in PEI, and recruited cells were analyzed after 24 h (Fig. 4c). The strongest staining was observed for monocytes and DC, which strongly express heparan sulfate proteoglycans, a class of surface molecules preferentially bound by PEI (13), but some labeling was also observed with neutrophils and B cells. Thus, PEI elicits a mixed cytokine response and recruits APC into the peritoneal cavity that subsequently take up the antigen-adjuvant coformulation.

Adjuvant mechanism of systemically administered PEI

IL-1β is a hallmark of inflammasome activation and is released rapidly after intraperitoneal PEI injection (Fig. 4a), a result consistent with the *in vitro* PEI activation of IL-1β, IL18 and caspase-1 in macrophages that we demonstrated previously (3). To determine the role of Nlrp3 signaling in the adjuvant activity of PEI after systemic administration, we immunized Nlrp3-deficient ($Nlrp3^{-/-}$) mice subcutaneously with gp140 in the presence or absence of PEI and quantified antigen-specific antibody responses. Total IgG responses were equivalent in wild-type (WT) and $Nlrp3^{-/-}$ mice (Fig. 5a), demonstrating that intact Nlrp3 function is not required for elicitation of adaptive immune responses by PEI. However, when antibody isotypes were evaluated, $Nlrp3^{-/-}$ mice showed a >10-fold increased level of specific IgG2c compared to WT, resulting in a substantially reduced IgG1/IgG2c ratio (Fig. 5b). These data indicate that similar to the mucosal mechanism of action of PEI, Nlrp3 inflammasome function is not required for global PEI adjuvant activity, but that its activation modulates adaptive immunity toward a T_h2-biased response.

We previously demonstrated that mucosally administered PEI released cellular DNA that triggered immune responses via an Irf3-mediated pathway (3). To investigate whether this also applied to systemically administered PEI, we compared gp140-specific IgG1 responses in $Irf3^{-/-}$ and WT mice. By contrast with mucosal administration, systemically administered PEI yielded equivalent responses in both Irf3-deficient and control mice (Supplementary Figure 1, available at *International Immunology* Online), indicating that Irf3 is not required for PEI adjuvantation via this route.

PEI coformulation with CpG ODNs augments the immune response and biases toward T_h1

We hypothesized that PEI might protect and deliver CpG ODNs to their endosomally localized receptor, TLR9 (14), and thus synergistically combine PEI adjuvanticity with a strong TLR-based T_h1 signal. To test this concept, we subcutaneously

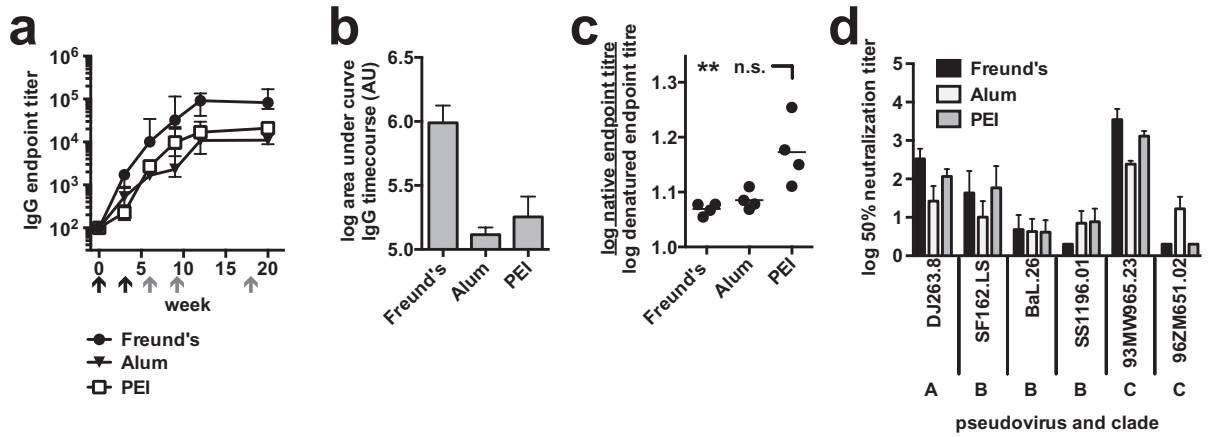

Fig. 3. Adjuvant activity of PEI in rabbits and neutralizing capacity of rabbit immune sera. New Zealand White rabbits (n = 4/group) were subcutaneously immunized five times with 50 μg gp140 (first immunization) or 20 μg gp140 (all subsequent immunizations), either alone or combined with FCA/FIA, 10 mg aluminium salts (alum) or 200 μg PEI; The first two immunizations included adjuvant (↑), while all subsequent immunizations contained only antigen (↑). (a) Timecourse gp140-specific serum pan IgG endpoint titers. (b) Week 0–20 area under $\log_{10}$-transformed serum pan IgG timecourse curve as a measure of the total immunogenicity of the formulations. (c) Log_{10} titer ratio of individual sera binding to native versus denatured gp140. Mean ratios for week 9, 12 and 20 samples per animal are displayed and were analyzed using the Kruskal–Wallis test followed by a Dunn's multiple comparison test. (d) HIV-1 pseudovirus neutralization assay using TZM-BL cells. Rabbit immune sera were tested for neutralization capacity against a series of pseudovirus preparations bearing heterologous HIV-1 wild-type isolate Envs. Data are presented as mean $\log_{10}$-transformed ± SEM. AU, arbitrary unit; $^{**}P < 0.01$.

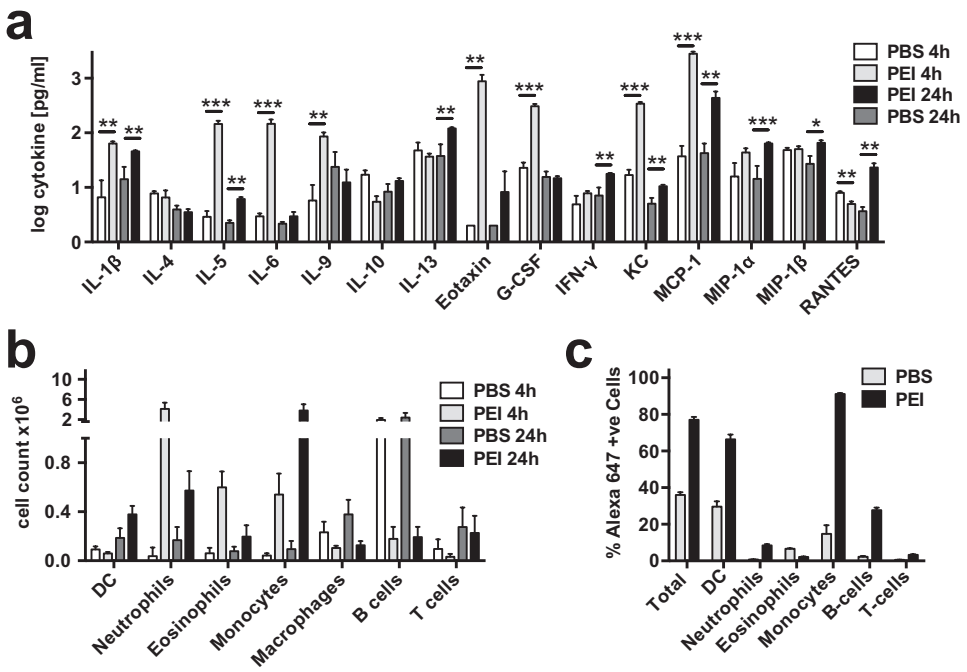

Fig. 4. Acute cytokine/chemokine responses, cell recruitment and cellular antigen uptake in BALB/c mice after intraperitoneal PEI injection. (a) Peritoneal lavage cytokine induction ($\log_{10}$-transformed data) 4 or 24 h after peritoneal PEI injection (80 μg, n = 5, ANOVA/Bonferroni test). (b) Peritoneal leukocyte recruitment 4 or 24 h after intraperitoneal PEI injection. Peritoneal lavage leukocyte populations were identified by flow cytometry with appropriate labeling and gates. (c) Uptake of Alexa-647-labeled OVA (10 μg) 24 h after intraperitoneal coinjection with 70 μg PEI (n = 5). Cytokine secretions were analyzed by multiple t-tests (one per cytokine and timepoint) adjusted with the Holm-Sidak correction. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

immunized mice with gp140 alone or combined with CpG ODNs, PEI or PEI-CpG ODN coformulations at different nitrogen to phosphorus (N/P) ratios (15). To characterize the additional activity of CpG ODNs combined with PEI, we used low doses of PEI and CpG ODNs alone that gave minimal systemic adjuvant activity after priming. We found that PEI-CpG coformulated at an N:P ratio of 15 (25 μg PEI + 12.4 μg CpG ODN 1018) induced ~10-fold enhanced antigen-specific

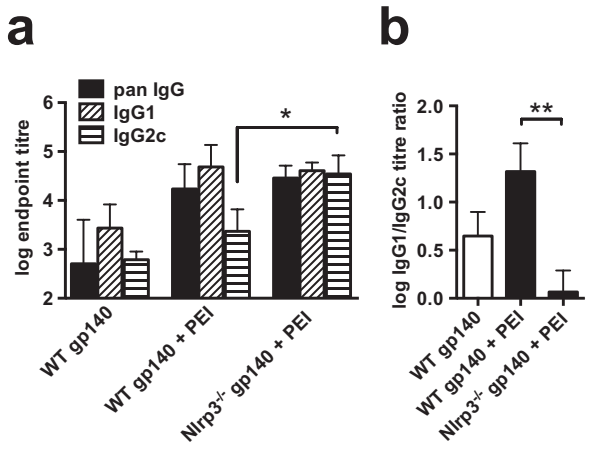

Fig. 5. Nlrp3 is not required for adjuvant activity of PEI but influences its immune bias. (a) gp140-specific serum immunoglobulin responses of C57Bl/6 WT or NLRP3$^{-/-}$ mice (n = 5/group). Mice received two subcutaneous immunizations with 2 μg gp140 in 100 μg PEI or in PBS alone. (b) IgG1/IgG2c titer ratio of the results shown in (a). Log_{10}-transformed titer data were analyzed by ANOVA followed by Dunnett's post-test. Data are presented as mean log_{10}-transformed ± SEM; *P < 0.05; **P < 0.01.

serum IgG1 and IgG2a responses after a single immunization compared to adjuvantation with PEI alone (Fig. 6a–c), demonstrating the synergistic action of PEI-CpG ODN coformulations after a single immunization. A second immunization revealed the adjuvant effect of PEI alone + antigen, and there was a non-significant trend toward increased antigen-specific IgG1 responses at the PEI-CpG N:P ratio of 15 (Fig. 6d). PEI-CpG combinations robustly increased antigen-specific IgG2a responses after the second immunization, with the N:P ratio of 15 yielding a significant (P < 0.05) increase compared to the group receiving PEI alone + antigen (Fig. 6e). However, the synergistic action of PEI and CpG ODN became less pronounced after the boost immunization. The IgG1/IgG2a titer ratios derived from PEI + CpG were decreased in comparison to PEI alone (Fig. 6f), implying a T_h1 shift upon coformulation of PEI with CpG ODNs. To confirm the T_h bias induced by the adjuvants *in vivo*, mice were immunized with gp140 alone, with CpG ODNs or PEI or a coformulation of PEI and CpG at an N:P ratio of 15. Two weeks after the final immunization of a homologous prime-boost regime, splenocytes were restimulated with overlapping peptide pools spanning the N-terminal (C1-C3) or C-terminal (C3-MPER) regions of HIV-1 gp140. Specific activity quantified in an IFN-γ ELISpot assay showed

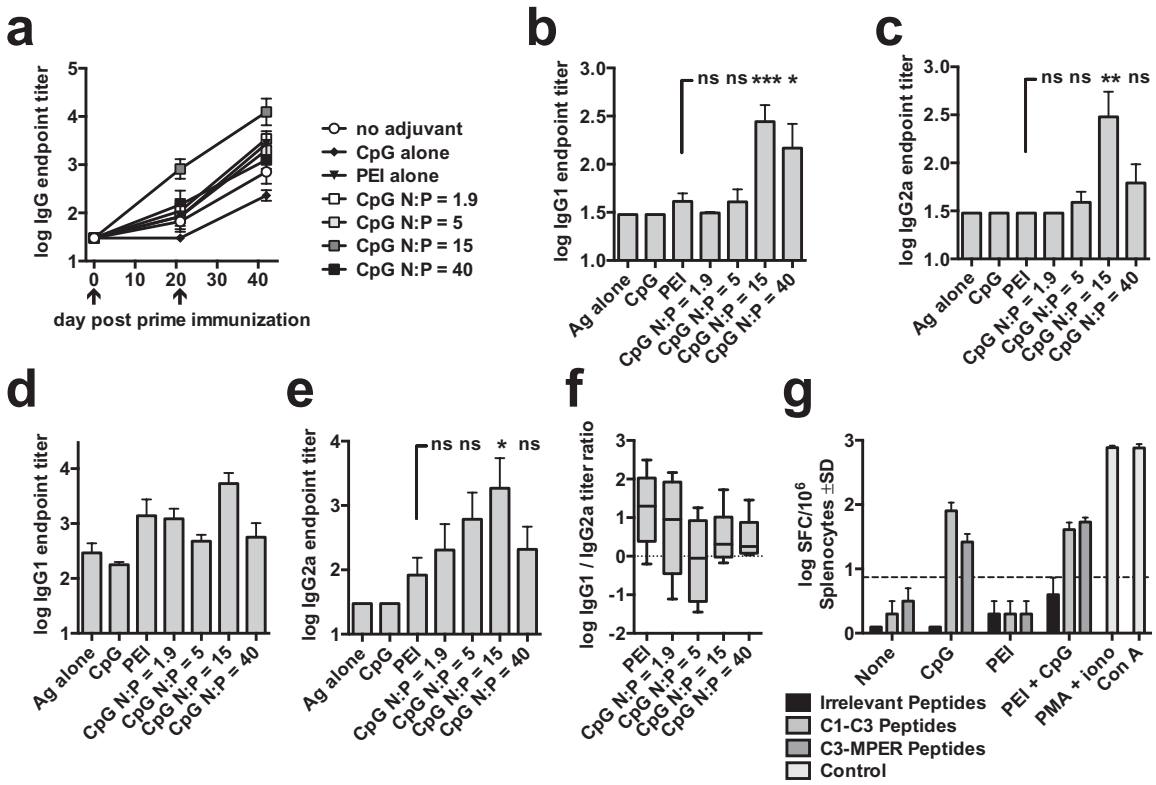

Fig. 6. Coformulation of PEI with CpG ODN modulates their adjuvant activity. (a–f) BALB/c mice (n = 5/group) received subcutaneous immunizations with 6 μg gp140 alone or combined with 100 μg CpG ODN 1018 alone (CpG), 25 μg PEI alone (PEI) or polyplexes composed of 25 μg PEI with 100 μg CpG (N:P ratio of 1.9), 37 μg CpG (N:P ratio of 5), 12.4 μg CpG (N:P ratio of 15) or 4.6 μg CpG (N:P ratio of 40) at day 0 and were boosted with protein alone at day 21. (a) Timecourse of gp140-specific serum IgG responses of immunized mice; (b) Week 3 (post-prime) serum IgG1 responses; (c) Week 3 serum IgG2a responses; (d) Week 6 (post-boost) serum IgG1 responses; (e) Week 6 serum IgG2a responses; (f) Week 6 log IgG1/IgG2a titer ratio. Log_{10}-transformed titer data were analyzed by ANOVA followed by Dunnett's post-test. (g) BALB/c mice (n = 3/group) were immunized twice subcutaneously with 20 μg gp140 alone or combined with 50 μg CpG ODN 2395 or 100 μg PEI or a coformulation of the two adjuvants corresponding to a N:P ratio of 15. Two weeks after the final immunization, splenocytes were restimulated with peptide pools spanning the N-terminal (C1-C3) or C-terminal (C3-MPER) region of HIV-1 gp140. Specific responses were quantified in an IFN-γ ELISpot assay. Data are presented as mean log_{10}-transformed ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

responses above threshold for mice immunized with CpG alone or PEI-CpG combined, but not with PEI alone (Fig. 6g). Taken together, these data demonstrate that, in comparison to PEI alone, PEI-CpG combination induced T_h1-biased responses that were optimal at N:P = 15 and synergistically elicited higher antigen-specific antibody responses than the isolated components.

Discussion

Here, we demonstrate that various forms of PEI act as potent systemic adjuvants that induce higher titers of antibody against natively folded antigen compared to alum. In combination with our previous results showing mucosal immune stimulating activity (3), these data confirm that potent adjuvant activity is an intrinsic and generalizable property of this family of molecules. Further characterization of branched 25 kDa PEI revealed that it drives a mixed T_h1/T_h2-type adaptive immune response if applied systemically, with robust antibody production in mice and rabbits. The induction of high antibody titers against the weakly immunogenic HIV-1 Env glycoprotein is promising, particularly since PEI-adjuvanted immunization generates antibodies that are more reactive with the native antigen compared to those elicited by the gold-standard experimental adjuvant FCA/FIA, which is too reactogenic for human use, and the clinically acceptable adjuvant alum. This is of particular relevance to the induction of neutralizing antibodies against HIV-1, as broadly neutralizing antibody epitopes are conformational in nature and require conservation of secondary, tertiary and in some cases, quaternary antigenic structure (10, 11, 16). The equivalent HIV-1 pseudovirus neutralization capacity of sera from rabbits immunized with gp140 in PEI or FCA/FIA bodes well in this respect, since FCA/FIA yielded higher absolute antigen-specific IgG titers but had lower proportional native gp140 binding activity. Although rabbits were immunized only twice in the presence of adjuvant and thereafter three times without (Fig. 3), our data confirm previous results showing a preferential antibody induction against the native antigen after mucosal immunization using PEI as adjuvant (3), suggesting that initial immunizations may play a pivotal role in determining the quality of the resulting antibody response. Despite the low power of the rabbit study, this effect reached statistical significance when comparing immune sera of PEI-immunized rabbits with those of FCA/FIA-immunized rabbits. Therefore, PEI might be an appropriate adjuvant for future HIV-1 vaccine strategies, particularly in combination with next generation protein antigens with stringent requirements for preservation of conformation to induce functional antibody dependent cell-mediated cytotoxicity (ADCC)-inducing and/or broadly cross-reactive HIV-1 neutralizing antibodies, but requiring adjuvant-mediated triggering of IgG production and affinity maturation.

The gene transduction activity of PEI depends upon delivery to, and destabilization of, endosomal membranes (17, 18), an effect that is associated with Nlrp3 inflammasome activation (19–21). However, despite the fact that PEI induced IL-1β release, a hallmark of inflammasome activation in our experiments [Fig. 4a and (3)], the global adjuvant effect was independent of the Nlrp3 inflammasome. Alum activates the inflammasome similarly to PEI, but in line with our findings several investigators have observed that inflammasome activation is not a requirement for alum adjuvant activity (22–24). However, the presence of Nlrp3 did influence the isotype of antigen-specific antibodies produced by PEI adjuvantation, strongly inferring an immune-modulating effect of the inflammasome toward a T_h2-type response. By comparison with PEI, alum induced a comparable acute chemokine secretion profile (MCP-1, Eotaxin, KC) in the peritoneal inflammation model (12). Although the recruited cell types following PEI-adjuvanted immunization will contribute to the cytokines/chemokines detected in peritoneal exudate, local immune and stromal cell-released cytokines play a critical role in orchestrating initial recruitment of immune cells. Accordingly, after alum-adjuvanted immunization, antigen-restimulated lymph node cells secreted IL-4, IL-5 and IL-10 (12), while in our experiments, PEI-adjuvanted immunization did not lead to significantly enhanced IL-4 and IL-10 secretion, indicating a weaker T_h2 immune bias than that induced by alum.

IFN signaling via Irf3 played a central role in the adjuvant activity of mucosally administered PEI via PEI-mediated release of cellular dsDNA (3). By contrast, here, we find that Irf3-deficient mice responded equivalently to their wild-type controls. This mechanistic dependence on route of delivery is at present unclear. We hypothesize that different environmental and/or tissue-specific factors will influence adjuvant activity. Unlike systemic routes of immunization, the upper airways are not a sterile compartment and resident microorganisms will most likely influence immune cell recruitment and activation. Accordingly, monocytes are one of the major cell populations that are recruited after peritoneal PEI application and take up large amounts of coformulated antigen (Fig. 4b and c) but appear to play a minor role after mucosal PEI administration (3). Thus, although PEI is likely to release similar damage-associated molecular pattern molecules independent of the site of administration, their functional significance may vary in a context-dependent manner.

The mixed T_h1/T_h2 response induced by PEI is adequate for eliciting high titer antibody responses but is unlikely to be optimal for coinduction of cytotoxic T cells that require a T_h1 cytokine environment (3). The finding that coformulation of PEI with the TLR ligand CpG ODNs synergistically increases the magnitude of the adaptive immune response and biases the response toward T_h1 is therefore highly relevant and suggests that PEI might be a useful platform for delivery of TLR3, -7/8 and -9 ligands to their site of action, the endosomal compartment of APC. Although there is compelling evidence that the polymer PEI can bind and compact both nucleic acids (6) and protein antigens (25), the potential formation, size and stability of ternary PEI-protein antigen-CpG ODN complexes requires further characterization in future studies. A PEI-CpG ODN-induced T_h1 bias might be particularly beneficial in the context of HIV-1 vaccine development to modulate the humoral response away from systemic IgA induction (26), which has been shown to increase the risk of HIV-1 acquisition in the RV144 vaccine trial (27), likely by dampening IgG-mediated ADCC responses (28). Targeted delivery of TLR ligands can also be beneficial from a safety point of view. Adjuvants based on soluble TLR ligands have been associated with systemic adverse effects (29, 30). Use

of PEI as delivery system for these agonists may focus the immunopotentiating effect to a minor local proportion of APC and thereby prevent systemic effects. Formal toxicity studies in animal models and reactogenicity profiles at clinically relevant adjuvant and antigen doses in clinical applications will be required to further characterize the tolerability of PEI coformulations for human vaccine use. Finally, since PEI is a soluble polymer, coformulation with antigen and TLR ligands needs only mixing, avoiding the more complicated procedures required for oil–water emulsions.

PEI's potent systemic adjuvant activity in combination with its unique capability to induce antibody responses against the non-denatured secondary structure of the antigen suggests that further preclinical development as a candidate adjuvant for human use is warranted.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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