U.S. Department of Veterans Affairs

Public Access Author manuscript

Acta Biomater. Author manuscript; available in PMC 2019 March 01.

Published in final edited form as:

Acta Biomater. 2018 March 01; 68: 168–177. doi:10.1016/j.actbio.2017.12.040.

“A poly(beta-amino ester) activates macrophages independent of NF- κ B signaling”

Neil M. Dold¹, Qin Zeng¹, Xiangbin Zeng¹, and Christopher M. Jewell^{1,4,*}

¹Fischell Department of Bioengineering, University of Maryland, 8228 Paint Branch Drive, College Park, MD, 20742

²United States Department of Veteran Affairs, 10 North Greene Street, Baltimore, Maryland 21201

³Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, MD 21201

⁴Marlene and Stewart Greenebaum Cancer Center, 22 South Greene Street, Baltimore, MD 21201

Abstract

Nucleic acid delivery vehicles are poised to play an important role in delivering gene therapy for vaccines and immunotherapies, and in delivering nucleic acid based adjuvants. A number of common polymeric delivery vehicles used in nucleic acid delivery have recently been shown to interact with immune cells and directly stimulate immunogenic responses, particularly in particle form. Poly(beta-amino esters) were designed for nucleic acid delivery and have demonstrated promising performance in a number of vaccine and therapeutic studies. Yet, little work has characterized the mechanisms by which these polymers activate immune cells. Here we demonstrate that a poly(beta-amino ester) activates antigen presenting cells in soluble and particulate forms, and that these effects are independent of TLR signaling pathways. Moreover, we show the polymers induce activation independent of NF- κ B signaling, but do activate IRF, an important innate inflammatory pathway. New knowledge linking physicochemical features of poly(beta-amino esters) or other polymeric carriers to inflammatory mechanisms could support more rational design approaches for vaccines and immunotherapies harnessing these materials.

Graphical Abstract

*To whom correspondence should be addressed: Prof. Christopher Jewell, Fischell Department of Bioengineering, 2212 Jeong H. Kim Engineering Building, College Park, MD 20742, Office: 301-405-9628, Fax: 301-405-9953, cmjewell@umd.edu, Web: jewell.umd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURES

The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Keywords

poly(beta-amino ester); macrophage and dendritic cell; vaccine and immunotherapy; microparticle and nanoparticle; immune response and inflammation

1. INTRODUCTION

Biodegradable polymer delivery vehicles for gene therapies that provide protection against nucleic acid degradation, controlled release, and passive targeting have been intensely studied to enhance efficacy and durability of nucleic acid vaccines and therapies. These approaches are generally designed to enhance the expression of proteins that the immune system should target, or to act as stimulatory signals by mimicking the nucleic acid structures of viruses or bacteria, which bind immunostimulatory receptors such as toll-like receptors (TLRs) [1, 2]. Poly(beta-amino esters) (PBAEs) are biodegradable polymers that can complex with nucleic acid cargos through electrostatic condensation owing to the cationic nature of the polymer's amine groups and anionic phosphate backbone of nucleic acids. Together, these cationic and anionic moieties can self-assemble into structures such as layered films on microneedles [3] and nanoparticle complexes. PBAEs are known to promote cellular uptake and endosomal escape into the cytosol via the proton sponge effect [4], which could promote interactions with TLRs that are localized to endosomes or to other similar receptors (e.g. RIG-I, MDA5 [5]) localized in the cytosol. PBAEs also can be synthesized with varying features (e.g. molecular weight, end groups, etc.) that may affect intracellular delivery – i.e. transfection efficiency – and interactions with individual cell types [4, 6-11].

Because nucleic acid cargos can have off-target effects including immune stimulation [12, 13] and because PBAEs can exhibit intrinsic immunogenicity in certain forms [10, 11], it is vital to understand the ability of these materials to either mitigate or enhance pro-inflammatory signaling. The immunogenic properties of a polymeric carrier can complicate vaccine design because the carrier itself impacts the response to the antigen – the biological target of the immune system – or to other vaccine components. Such a response might be undesirable in the case of vaccines and immunotherapies aimed at attenuating autoimmune disease. At the same time, immunostimulatory effects from carriers can be desirable when controlled or predictable, for example, in immunotherapies and vaccines targeting cancer or infectious disease. Understanding how specific properties of materials drive intrinsic immunogenic effects could allow faster, more rational strategies for engineering carriers that help actively direct an immune response [14, 15].

Antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages are innate immune cells that surveil the body for pathogens and damaged tissues. These cells are important players in the response to biomaterial vaccine carriers. When these cells encounter immunostimulatory signals – such as biological patterns that appear in pathogens, but not in humans – APCs coordinate the immune response by phagocytosing and presenting antigen molecules to lymphocytes in lymph nodes, which initiate the adaptive immune response [16]. Consequently, polymeric delivery vehicles that activate specific functions in APCs are valuable tools in targeted stimulation of the immune system for vaccines and immunotherapies that require pro-inflammatory responses.

Particles are attractive vehicles for vaccine delivery because they can be formulated into different sizes and shapes that generally encourage interactions with APCs through phagocytosis [17, 18], and they can promote gene transfection [19]. For example, DNA encoding antigen for a particular pathogen can be used to prime the immune system to attack that antigen. Moreover, polymeric particles can directly enhance activation of immune pathways via intrinsic immune effects through a range of inflammatory pathways including: the inflammasome [20], toll-like receptors (TLRs) and MyD88 signaling [21], interferon-regulatory factor (IRF)-dependent signaling [22], and complement [23, 24] – some of which signal through or in cooperation with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factor signaling [1, 25, 26].

Recently, much attention has been paid to immunotherapy using RNA interference (RNAi), which can be accomplished through delivery of small interfering RNAs (siRNA) [27, 28]. These short, double stranded RNAs cause degradation of target mRNAs through the RISC complex, and they generally lead to lower expression of the associated protein. Therefore, RNAi could be used to attenuate activity of immune checkpoint molecules (e.g. CTLA-4, PD-1, TIM-3), which are overexpressed in tumor microenvironments to suppress immune cell activity against tumors [29]. Silencing these checkpoint molecules could restore function to immune cells and promote tumor killing.

Our lab has previously used DCs isolated from mice to show that PBAEs exhibit intrinsic immune function when in particulate form following complexation with a polyanion, poly(sodium 4-styrenesulfonate); PBAE degradation [10] and molecular weight [11] played roles in the nature of the immune response to these particles. Because PBAEs were designed and characterized as nucleic acid delivery vehicles [4, 6], here we extend our studies to characterize the intrinsic immunogenicity of these polymers during delivery of siRNA in an *in vitro* model of immune activation, RAW264.7 mouse macrophages. RAW264.7 cells are a popular *in vitro* model for innate immune responses to biomaterials because they are a well-established cell line that exhibits behaviors similar to primary APCs [30]; they are also recommended for use in international standards for implanted biomaterials testing [31] and could serve as a screening tool to develop standard approaches for assessing the immunological features of new synthetic carriers. Moreover, macrophages represent an alternative therapeutic target – in comparison to approaches that focus on delivering particles to DCs and gene therapies to lymphocytes – for particles containing nucleic acids that stimulate immune responses against diseases like cancer because they reside in all tissues

and have unique capabilities in phagocytosing and clearing particles and/or cellular debris [32, 33].

We investigate the intrinsic ability of a PBAE to enhance pro-inflammatory pathways in both soluble and particle form. For particle studies, we show that a PBAE and siRNA can be assembled into nanoparticle complexes with tunable size and charge. In both particulate and free form, the PBAE activates the macrophages. Interestingly, we provide data suggesting this activation is different from that arising through TLRs, and we show that activation is independent of common inflammatory immune pathways including NF- κ B.

2. MATERIALS AND METHODS

2.1. Materials

PBAE ($M_n = 1022$ Da, $M_w = 1347$ Da, PDI = 1.32) was synthesized in a Michael-type addition reaction using standard methods previously described [10]. The NC1 control dicer-substrate siRNA (DsiRNA) duplex (5'-CGUUAUUCGCGUAUAUACGCGUAT-3') and CpG ODN were purchased from Integrated DNA Technologies. Dulbecco's modified eagle medium (DMEM, Gibco) and heat-inactivated fetal bovine serum (FBS) were purchased from ThermoFisher Scientific and Corning, respectively. Antibiotics (Normocin, Zeocin, and Blasticidin), low molecular weight polyinosinic-polycytidylic acid (polyIC, tlr-picw) and R848 were purchased from Invivogen. Lipopolysaccharide (LPS) and sodium acetate buffer were purchased from Sigma-Aldrich. Fluorescent antibodies for maturation markers (PE CD40, clone 3/23; APC CD80, clone 16-10A1; PE-Cy7 CD86, clone GL1) and blocking antibodies for Fc receptor blocking (clone 2.4G2) were purchased from BD Biosciences. All ELISA sets were purchased from BD Biosciences.

2.2. Cells

RAW264.7 cells and RAW-Dual (IRF-Lucia/KIN-[MIP-2]SEAP) cells were purchased from ATCC and Invivogen, respectively. Both cell types were maintained in DMEM containing 4.5g/l glucose and 4mM l-glutamine, and the media for both cell types was supplemented with 10% FBS. For RAW-Dual cells only, antibiotics were added per the supplier's recommendation with Normocin at 100 μ g/ml for all passages and Zeocin every other passage at 200 μ g/ml to maintain selective pressure on the reporters which are activated through the NF- κ B and IRF pathways. Cells were grown in tissue culture-treated polystyrene flasks, while experimental treatments in 96-well plates were performed using non-treated polystyrene. RAW-Dual assays were performed without either antibiotic following a passage containing both Normocin and Zeocin.

Mouse TLR reporter cells (HEK-Blue mTLR3, HEK-Blue mTLR7, and HEK-Blue mTLR9) were purchased from Invivogen and cultured in DMEM with 10% FBS. Each cell type is stably transfected with one mouse TLR (TLR4, TLR7, and TLR9 respectively) and a secreted embryonic alkaline phosphatase (SEAP) reporter that is activated through the NF- κ B pathway. During growth, Normocin and penicillin-streptomycin were added per the supplier's protocol and selection media contained an additional antibiotic (Zeocin and Blasticidin) in to maintain the TLR and reporter plasmids. Cells were passaged in PBS

without trypsinization and plated in 96-well plates over TLR agonist and polymer treatments in HEK-Blue detection media purchased from Invivogen.

2.3. Nanoparticle synthesis and physicochemical analysis by size and zeta potential measurements

Complexes were assembled by mixing PBAE and NC1 DsiRNA in 25mM sodium acetate buffer at pH 5. Components were mixed at 1:1 v:v ratios, and the w:w ratio was varied by diluting PBAE. Each batch of particles was assembled by mixing 25pL of PBAE solution with 25pL of DsiRNA solution. Prior to mixing, the concentration of the DsiRNA solution was fixed at 40µg/mL, while the highest concentration of PBAE solution was 2mg/mL for the 50:1 ratio complexes; decreasing concentrations were prepared by diluting the PBAE solution accordingly prior to mixing. Immediately following complex assembly for each batch, dynamic light scattering (DLS, size) and electrophoretic light scattering (ELS, zeta potential) were performed using a Malvern Zetasizer Nano ZS90. Measurements were performed in disposable 50µL cuvettes and in capillary cells for DLS and ELS, respectively. ELS measurements were conducted using the diffusion barrier technique [34]; briefly, immediately following DLS, sample (up to 50µL) was transferred from the disposable cuvette and concentrated at the bottom of a capillary cell previously filled with the sodium acetate buffer using a flexible gel-loading pipette tip.

2.4. Quantification of macrophage cell line activation by flow cytometry

RAW264.7 cells were passaged (no greater than P10) by cell scraping and seeded at 50,000-55,000 cells in 170µL per well for all experiments in 96-well plates on top of prepared treatments (e.g. complexes, soluble polymer or RNA, TLR agonist) delivered in 30µL of 25mM sodium acetate buffer per well. At 24 and 48 hours cells were detached using Cellstripper (Corning) and resuspended in PBS containing 1% bovine serum albumin (Sigma-Aldrich). After blocking the Fc receptors, cells were stained with fluorescent antibodies for surface activation markers (i.e. CD40, CD80, and CD86) and with a viability dye (DAPI). Flow cytometry (BD FACSCanto II) was used to determine relative levels of activation through analysis using FlowJo (v.10.2, TreeStar, Ashland, OR). Cell supernatants were saved for analysis by ELISA.

2.5. Immune pathway characterization in RAW-Dual reporter cell line

RAW-Dual cells were prepared and treated in the same manner as RAW264.7 cells described above. Following 24 or 48 hour incubation, supernatants were collected and aliquots were tested for the secreted Lucia luciferase and SEAP reporters (IRF and NF-κB, respectively) using Invivogen's QUANTI-Luc and QUANTI-Blue detection reagents; values were recorded for luminescence and absorbance per manufacturer's protocol.

2.6. TLR pathway characterization in HEK-Blue mTLR cell lines

HEK-Blue (mTLR3, mTLR7, and mTLR9) reporter cells were passaged and treated in HEK-Blue Detection medium (Invivogen) with soluble PBAE, complexes, and controls in the same manner as the RAW264.7 and RAW-Dual cells as described above. The SEAP reporter induced by the TLR in each of the three cell lines was quantified using HEK-Blue Detection

medium by incubating cells with the treatments overnight. Relative levels of the reporter expression were quantified via absorbance measurements per manufacturer's protocol.

2.7. ELISAs

Cell supernatants were collected when processing cells for flow cytometry experiments. ELISAs against IL-6 and IL-10 were performed according to manufacturer's instructions (BD Biosciences) at 5x dilutions, except for LPS-treated samples in the IL-6 ELISAs, which were diluted 10x. Standard curves were generated using 4 or 5 parameter logistic models in GraphPad Prism 7 or in SoftMax Pro 4.8.

2.8. Statistical analysis

One-way ANOVAs were performed along with pairwise comparisons using the Tukey method in Minitab 17.2. A p-value less than 0.05 indicated significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All error bars are reported as standard error of the mean (S.E.M.).

3. RESULTS

3.1. The PBAE:RNA ratio controls size and charge of resulting complexes

To avoid sequence-specific RNAi effects when examining the intrinsic immunogenicity of PBAE and RNA, we selected a commercially available DsiRNA, NC1, which does not target any part of the mouse or human transcriptome [35]. This allowed isolation of the immunogenic effects of the PBAE. PBAE (Figure 1A) and DsiRNA were assembled into particles at defined PBAE:RNA w:w ratios (Figure 1B) ranging from 50:1 to 1:1; particles were immediately characterized by measuring the diameter, zeta potential, and polydispersity. Over this range, nanoparticle complexes formed exhibiting mean diameters from 240nm and 503nm (Figure 1C) with mean zeta potentials from -13.1 mV to $+22.2$ mV (Figure 1C), and particles were narrowly or moderately disperse ($0.036 < \text{average PDI} < 0.131$) (Figure S1). At the most extreme ends of the ratios tested, complexes were smaller and more highly charged.

3.2. PBAE activates macrophages in both free and complexed form

In our lab's previous studies, free PBAE did not activate primary DCs, whereas PBAE particles did cause DC activation [10, 11]. Thus, we tested these same questions using macrophages. RAW264.7 cells in non-adherent 96-well plates were treated with soluble PBAE ranging from $0.5 \mu\text{g/mL}$ to $4 \mu\text{g/mL}$ and with PBAE-NC1 complexes delivered at $2 \mu\text{g/mL}$ and $3 \mu\text{g/mL}$. Complex treatments were matched to equivalent soluble PBAE dose using varying PBAE:RNA ratios (Table 1). Complex ratios for cell treatments were selected to cover the range of observed positive and negative complex charges.

At 24 and 48 hours following treatment, the cells receiving PBAE treatments were compared to cells receiving control treatments of buffer (negative control) or specific TLR agonists (positive control). Across all of these conditions and doses, we did not observe trends pertaining to toxicity at either 24 or 48 hours, as indicated by membrane leakiness assessed by DAPI staining (Figure 2).

We next assessed activation using flow cytometry to measure surface activation makers over time. In these studies, both soluble PBAE and PBAE-RNA complexes caused activation by 24 hrs (Figure 3A-C, Figure S2A-C). We observed statistically significant increases for all three makers assessed – CD40, CD80, and CD86, and these were dose dependent. We were not able to draw any statistically significant conclusions regarding differences in activation caused by free PBAE compared with PBAE complexes at the same dose. Activation levels were comparable to or higher than the levels induced by TLR7/8a (R848), but lower than the more potent TLR4a (LPS). Compared with 24 hrs, activation generally increased by 48 hours (Figure 3D-F, Figure S2D-F).

We also observed that RAW264.7 cells did not mature in response to the TLR3a, polyIC, but were activated by TLR4a (LPS) and TLR7/8a (R848), as just mentioned (Figure 3). With respect to structure, LPS, R848, and the highest doses of PBAE each caused significant changes in cell morphology associated with activation (Figure 4). These included spreading, culture density, and intracellular bodies – whereas polyIC and buffer controls did not exhibit these changes. As expected, treatment with DsiRNA alone, which can act as a TLR agonist if not structurally modified to prevent RNA-TLR interactions [13], also did not induce maturation.

3.3. PBAE does not induce IL-6 or IL-10 secretion

We next examined cytokine secretion from cells treated with each PBAE formulation as an initial indicator of the mechanism or type activation that a PBAE might trigger. Supernatants from treated RAW264.7 cells were analyzed by ELISA for IL-6 and IL-10. A primary characteristic of IL-6 is early indication of inflammatory pathways during wounds, disease, and infection [36]. IL-10 is a marker of alternative macrophage activation [37]. Despite clear induction of IL-6 secretion by the positive control (LPS), PBAE in either free or complexed form did not induce IL-6 production at either 24 or 48 hours (Figure 5A,B). R848 weakly induced IL-6 by 48 hrs. In the case of IL-10, both LPS and R848 induced IL-10 secretion, and as with IL-6, these levels were higher by 48 hrs (Figure 5C,D). However, neither type of PBAE treatment triggered IL-10 at 24 or 48 hrs (Figure 5C,D). Together, the results in Figures 3-5 suggest activation of RAW264.7 cells by PBAE is mediated by different pathways than those activated by TLR agonist such as LPS or R848.

3.4. PBAE does not stimulate TLR3, TLR7, or TLR9 in reporter signaling assays

Since synthetic polymer have previously been shown to enhance the immune response to TLR agonists, and some of these ligands are nucleic acids – including double stranded RNAs (TLR3), single stranded RNAs (TLR7), and CpG DNA (TLR9) [1], we tested if the stimulatory properties of PBAE and complexes might result from activation of TLR pathways. However, in reporter cell lines specific for activation of TLR3 (Figure 6A), TLR7 (Figure 6B), or TLR9 (Figure 6C), we did not observe any TLR signaling during treatment with soluble PBAE or PBAE complexed with NC1 RNA. In each case, specificity of the assays was confirmed by high activity levels measured during treatment with the appropriate TLR ligand – polyIC for TLR3, R848 for TLR7, CpG for TLR9 – and an absence of activity when cells were treated with irrelevant TLR ligands (negative controls) for each pathway.

3.5. PBAE activity involves IRF signaling, but is independent of NF- κ B

Because PBAE treatments did not activate the TLR receptors tested, and because they appear to activate macrophages via a different mechanism than LPS and R848, we quantified inflammatory transcription factor activity in macrophages to further probe the pro-inflammatory mechanism induced by the PBAE. We hypothesized PBAE would act through the NF- κ B and/or IRF families of transcription factors, which respond to a broad array of immune signals [25, 38]. To test this hypothesis we used RAW264.7 cells stably transfected with unique reporters for NF- κ B and IRF signaling. Cells were treated with soluble or complexed PBAE, or TLR ligands as positive (LPS, R848) and negative (polyIC) controls, then supernatants were collected for analysis. PBAE treatments that successfully matured macrophages in the studies above (Figures 3-4) did not induce NF- κ B signaling at either 24 or 48 hours, while the positive controls did activate this pathway (Figure 7A,B). No IRF activity was associated with any PBAE treatment after 24 hours (Figure 7C). In contrast, by 48 hrs, both free and complexed PBAE significantly activated IRF signaling (Figure 7D). When IRF was activated, reporter levels were comparable to stimulation with R848, but lower than LPS. Taken together, the mechanistic data in Figures 5-7 provide evidence that the inflammatory mechanism in PBAE-treated macrophages is at least partially distinct from those triggered by TLR agonists.

4. DISCUSSION

Our previous work with PBAEs in primary DCs isolated from C57BL/6J mice, and *in vivo* using this same strain, revealed stimulatory effects only when the PBAE was formulated in particles with an anionic polymer as a nucleic acid model [10, 11] rather than when PBAE was delivered alone. Building on these studies, we extended our investigations in this report of PBAE immunogenicity to an important macrophage cell line that could help support development of standards for screening immune-modulatory properties of new carriers.

Here, we showed that a PBAE can be formulated into nanoparticles with both negative and positive charges (Figure 1) by varying the w:w ratio of PBAE and functionally inert DsiRNA, similar to particles that are useful in functional nucleic acid delivery. These nanoparticle complex formulations may also contain some amount of free PBAE or DsiRNA because formation of the complexes depends on electrostatic interactions and the ratios of the complex components. Interestingly, in this setting, we discovered a PBAE can act as an immunostimulatory agent in both soluble and particle forms, not only in the particle form. This intrinsic immunogenicity could enable design of PBAE carriers that efficiently condense and deliver nucleic acid cargo (e.g. encoding vaccine antigens), while also helping to stimulate immune function against these antigens. Without condensation into a polyplex – even though the immunogenicity of the polymer may remain – the nucleic acid vaccine cargo is unlikely to enter the cell and be expressed. However, it is also important to note that RAW264.7 macrophages are immortalized from BALB/c mice, which could introduce strain-specific differences in immunological response compared to previous work in primary DCs [39, 40].

Although the PBAE used here clearly activated the macrophages, our observations suggest that the effects are mechanistically distinct from the TLR agonists we studied. One piece of

evidence is that LPS (TLR4) induced a unique change in morphology (Figure 4) combined with much higher expression compared to PBAE and all other treatments. In contrast, polyIC (TLR3) showed no signs of activating cells (other work indicates that this is expected in RAW264.7 with LMW polyIC, possibly due to low TLR3 expression [41]), while R848 (TLR7/8a) and PBAE induced moderate to high levels of activation of CD40, CD80, and CD86 (Figure 3). Furthermore, PBAE did not induce IL-6 or IL-10 expression (Figure 5).

After showing that PBAE and PBAE-RNA complexes do not induce TLR activity (Figure 6), we hypothesized that the PBAE immunogenicity would be regulated through the IRF and/or NF- κ B transcription factors due their broad roles in regulating many of the most common pro-inflammatory signaling pathways [25, 38]. Intriguingly, PBAE showed no sign of signaling through NF- κ B, while IRF was activated at 48 hours (Figure 7). Thus, perhaps the strongest evidence for different mechanisms of activation between PBAE and TLR agonists is that while PBAE, LPS, and R848 all activated macrophages (Figure 3, Figure S2), LPS and R848 activated their expected TLR pathways, which signal through IRF and NF- κ B. The PBAE did not activate TLR or NF- κ B signaling, but it did activate IRF at 48 hours.

Although our approach represents only one means of measuring IRF and NF- κ B induction, the analysis above suggests the PBAE activates macrophages by some means other than pathogen recognition receptors such as TLRs, which tend to signal through one or both of those transcription factors. It will be important to establish the inflammatory pathway activated by PBAE because different activation pathways have distinct functions in the context of disease. For example, some activated macrophages are involved in repairing tissue or resolving inflammation caused by wounds, while other activated macrophage phenotypes are involved in response to a pathogen or anti-tumor activity [16, 32].

There are also some open questions that may reveal additional pathways involved in activation. For example, we observed that macrophages are activated by 24 hours, but no IRF activity was measured until 48 hours, suggesting alternate or upstream pathways activated earlier may be important in the response to PBAE or other synthetic materials. Measurement of IL1- β could rule out or show interaction of PBAE with the inflammasome, which typically requires participation of NF- κ B inducers [26]. Another potential mechanism involves the complement receptor 3, CR3, which is expressed on RAW264.7 cells [30], and is involved in phagocytosis/opsonization of certain pathogens in a MyD88-independent manner precluding IRF and NF- κ B [42].

As mentioned above, immunological variation among mouse strains [40] and cell type (i.e. DCs versus macrophages) may contribute to differences in our findings between these two settings. Additionally, because RAW264.7 cells are immortalized, they may differ further in behavior from primary cells *in vitro* models. Ultimately, because distinctions from mouse to human immune systems are vast [43], cataloging the changes across pre-clinical models is of considerable interest to abate the frequent failure to translate candidate therapies to clinical successes. Fast, standardized methods to reliably assess the immunogenic properties of new materials and carriers *in vitro* could provide a means to help overcome this challenge.

5. CONCLUSION

By measuring activation markers, cytokines, and transcription factor activity, we have shown that a PBAE activates RAW264.7 cells – a common *in vitro* macrophage culture. Our findings are important because 1) new immunological adjuvant strategies are needed to enable more effective vaccines and immunotherapies and because 2) these types of pro-inflammatory effects should be taken into consideration when developing therapies with PBAEs or other synthetic materials that might cause unwanted inflammation, or that could allow tuning of inflammatory response. Additionally, the intrinsic immunogenicity of polymers and other therapeutic delivery vehicles remains under studied, and the structure-function relationships of the most common clinically used adjuvants (e.g. alum) are still being established [44, 45]. Although these findings should not be immediately extrapolated to all immunogenic synthetic carriers, they suggest pathways to investigate the mechanisms by which new carriers may modulate immune cell interactions, transfection efficiency, *in vivo* trafficking, and vaccine efficacy [8, 9, 46].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors acknowledge Shannon J. Tsai for assistance with polymer characterization. This work was supported in part by NSF CAREER Award # 1351688 and Alliance for Cancer Gene Therapy #15051543. C.M.J. is a research scientist at the VA Maryland Health Care System, Baltimore, MD. N.M.D.'s training was supported in part by the Partnership for Integrative Cancer Research between the National Cancer Institute and University of Maryland.

REFERENCES

- [1]. O'Neill LAJ, Golenbock D, Bowie AG, The history of Toll-like receptors - redefining innate immunity, *Nature reviews. Immunology* 13(6) (2013) 453–60.
- [2]. Zhang M, Hong Y, Chen W, Wang C, Polymers for DNA Vaccine Delivery, *ACS Biomaterials Science & Engineering* 3(2) (2017) 108–125.
- [3]. DeMuth PC, Min Y, Huang B, Kramer J.a., Miller AD, Barouch DH, Hammond PT, Irvine DJ, Polymer multilayer tattooing for enhanced DNA vaccination, *Nature materials* 12(4) (2013) 367–76. [PubMed: 23353628]
- [4]. Akinc A, Anderson DG, Lynn DM, Langer R, Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery, *Bioconjugate chemistry* 14(5) (2003) 979–88. [PubMed: 13129402]
- [5]. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT, PAMPs and DAMPs: signal 0s that spur autophagy and immunity, *Immunological reviews* 249(1) (2012) 158–75. [PubMed: 22889221]
- [6]. Lynn DM, Langer R, Degradable Poly(β-amino esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA, *Journal of the American Chemical Society* 122(44) (2000) 10761–10768.
- [7]. Anderson DG, Lynn DM, Langer R, Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery, *Angewandte Chemie - International Edition* 42(27) (2003) 3153–3158. [PubMed: 12866105]
- [8]. Kim J, Sunshine JC, Green JJ, Differential polymer structure tunes mechanism of cellular uptake and transfection routes of poly(β-amino ester) polyplexes in human breast cancer cells, *Bioconjugate chemistry* 25(1) (2014) 43–51. [PubMed: 24320687]

- [9]. Sunshine JC, Peng DY, Green JJ, Uptake and transfection with polymeric nanoparticles are dependent on polymer end-group structure, but largely independent of nanoparticle physical and chemical properties, *Molecular pharmaceutics* 9(11) (2012) 3375–83. [PubMed: 22970908]
- [10]. Andorko JI, Hess KL, Pineault KG, Jewell CM, Intrinsic immunogenicity of rapidly-degradable polymers evolves during degradation, *Acta biomaterialia* 32 (2015) 24–34. [PubMed: 26708710]
- [11]. Andorko JI, Pineault KG, Jewell CM, Impact of molecular weight on the intrinsic immunogenic activity of poly(beta amino esters), *Journal of Biomedical Materials Research - Part A* 105(4) (2017) 1219–1229. [PubMed: 27977902]
- [12]. Jackson AL, Linsley PS, Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application, *Nature reviews Drug discovery* 9(1) (2010) 57–67. [PubMed: 20043028]
- [13]. Ku SH, Jo SD, Lee YK, Kim K, Kim SH, Chemical and structural modifications of RNAi therapeutics, *Advanced Drug Delivery Reviews* 104 (2015) 16–28. [PubMed: 26549145]
- [14]. Tostanoski LH, Jewell CM, Engineering self-assembled materials to study and direct immune function, *Advanced Drug Delivery Reviews* 114 (2017) 60–78. [PubMed: 28392305]
- [15]. Andorko JI, Hess KL, Jewell CM, Harnessing Biomaterials to Engineer the Lymph Node Microenvironment for Immunity or Tolerance, *The AAPS Journal* 17(2) (2015) 323–338. [PubMed: 25533221]
- [16]. Jakubzick CV, Randolph GJ, Henson PM, Monocyte differentiation and antigen-presenting functions, *Nature Reviews Immunology* (2017).
- [17]. Zhang J, Saltzman M, Engineering biodegradable nanoparticles for drug and gene delivery, *Chemical Engineering Progress* 109(3) (2013) 25–30. [PubMed: 25374435]
- [18]. Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F, Immunological Principles Guiding the Rational Design of Particles for Vaccine Delivery, *ACS Nano* (i) (2017) acsnano.6b07343-acsnano.6b07343.
- [19]. Nguyen DN, Green JJ, Chan JM, Langer R, Anderson DG, Polymeric materials for gene delivery and DNA vaccination, *Advanced Materials* 21(8) (2009) 847–867. [PubMed: 28413262]
- [20]. Sharp F.a., Ruane D, Claass B, Creagh E, Harris J, Malyala P, Singh M, O'Hagan DT, Pétrilli V, Tschopp J, O'Neill L.a.J., Lavelle EC, Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome, *Proceedings of the National Academy of Sciences of the United States of America* 106(3) (2009) 870–5. [PubMed: 19139407]
- [21]. Uto T, Akagi T, Yoshinaga K, Toyama M, Akashi M, Baba M, The induction of innate and adaptive immunity by biodegradable poly(γ -glutamic acid) nanoparticles via a TLR4 and MyD88 signaling pathway, *Biomaterials* 32(22) (2011) 5206–5212. [PubMed: 21492934]
- [22]. Wegmann F, Gartlan KH, Harandi a.M., Brinckmann S.a., Coccia M, Hillson WR, Kok WL, Cole S, Ho LP, Lambe T, Puthia M, Svanborg C, Scherer EM, Krashias G, Williams a., Blattman JN, Greenberg PD, Flavell R.a., Moghaddam a.E., Sheppard NC, Sattentau QJ, Polyethyleneimine is a potent mucosal adjuvant for viral glycoprotein antigens, *Nat Biotechnol* 30(9) (2012) 883–888. [PubMed: 22922673]
- [23]. Reddy ST, van der Vlies AJ, Simeoni E, O'Neil CP, Swartz M.a., Hubbell J.a., Exploiting lymphatic transport and complement activation in nanoparticle vaccines, *European Cells and Materials* 14(SUPPL.3) (2007) 103–103.
- [24]. Thomas SN, van der Vlies AJ, O'Neil CP, Reddy ST, Yu SS, Giorgio TD, Swartz MA, Hubbell JA, Engineering complement activation on polypropylene sulfide vaccine nanoparticles, *Biomaterials* 32(8) (2011) 2194–2203. [PubMed: 21183216]
- [25]. Zhang Q, Lenardo MJ, Baltimore D, 30 Years of NF- κ B: A Blossoming of Relevance to Human Pathobiology, *Cell* 168(1–2) (2017) 37–57. [PubMed: 28086098]
- [26]. Afonina IS, Zhong Z, Karin M, Beyaert R, Limiting inflammation—the negative regulation of NF- κ B and the NLRP3 inflammasome, *Nature Immunology* 18(8) (2017) 861–869. [PubMed: 28722711]
- [27]. Jin H, Kim A, Miyata K, Kataoka K, Kim HJ, Recent progress in development of siRNA delivery vehicles for cancer therapy, *Advanced Drug Delivery Reviews* 104(9) (2016) 61–77. [PubMed: 27352638]

- [28]. Young SWS, Stenzel M, Jia-Lin Y, Nanoparticle-siRNA: A potential cancer therapy?, *Critical Reviews in Oncology/Hematology* 98 (2016) 159–169. [PubMed: 26597018]
- [29]. Pardoll DM, The blockade of immune checkpoints in cancer immunotherapy, *Nature Reviews Cancer* 12(4) (2012) 252–264. [PubMed: 22437870]
- [30]. Raschke WC, Baird S, Ralph P, Nakoinz I, Functional Macrophage Cell Lines Transformed by Abelson Leukaemia Virus, *Cell* 15(9) (1978) 261–267. [PubMed: 212198]
- [31]. A. International, ASTM F1903–10 Standard Practice for Testing For Biological Responses to Particles In Vitro, West Conshohocken, 2010.
- [32]. Komohara Y, Fujiwara Y, Ohnishi K, Takeya M, Tumor-associated macrophages: Potential therapeutic targets for anti-cancer therapy, *Advanced Drug Delivery Reviews* 99 (2016) 180–185. [PubMed: 26621196]
- [33]. Hashimoto D, Miller J, Merad M, Dendritic Cell and Macrophage Heterogeneity In Vivo, *Immunity* 35(3) (2011) 323–335. [PubMed: 21943488]
- [34]. Corbett JCW, Connah MT, Mattison K, Advances in the measurement of protein mobility using laser Doppler electrophoresis - the diffusion barrier technique, *Electrophoresis* 32(14) (2011) 1787–1794. [PubMed: 21769888]
- [35]. Kim D-H, Behlke M.a., Rose SD, Chang M-S, Choi S, Rossi JJ, Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy, *Nature biotechnology* 23(2) (2005) 222–226.
- [36]. Hunter C.a., Jones S.a., IL-6 as a keystone cytokine in health and disease, *Nature immunology* 16(5) (2015) 448–457. [PubMed: 25898198]
- [37]. Martinez FO, Gordon S, The M1 and M2 paradigm of macrophage activation: time for reassessment, *F1000prime reports* 6(3) (2014) 13–13. [PubMed: 24669294]
- [38]. Ikushima H, Negishi H, Taniguchi T, The IRF Family Transcription Factors at the Interface of Innate and Adaptive Immune Responses, *Cold Spring Harb Symp Quant Biol* LXXXVIII (2013).
- [39]. Passwell JH, Steward MW, Soothill JF, Inter-mouse strain differences in macrophage function and its relationship to antibody responses, *Clinical and experimental immunology* 17(1) (1974) 159–67. [PubMed: 4282849]
- [40]. Sellers RS, Clifford CB, Treuting PM, Brayton C, Immunological Variation Between Inbred Laboratory Mouse Strains: Points to Consider in Phenotyping Genetically Immunomodified Mice, *Veterinary Pathology* 49(1) (2012) 32–43. [PubMed: 22135019]
- [41]. Li N, Sun J, Benet ZL, Wang Z, Al-Khodori S, John SP, Lin B, Sung M-H, Fraser IDC, Development of a cell system for siRNA screening of pathogen responses in human and mouse macrophages, *Scientific Reports* 5 (2015) 9559–9559. [PubMed: 25831078]
- [42]. Hawley KL, Olson CM, Iglesias-Pedraz JM, Navasa N, Cervantes JL, Caimano MJ, Izadi H, Ingalls RR, Pal U, Salazar JC, Radolf JD, Anguita J, CD14 cooperates with complement receptor 3 to mediate MyD88-independent phagocytosis of *Borrelia burgdorferi*, *Proceedings of the National Academy of Sciences of the United States of America* 109(4) (2012) 1228–32. [PubMed: 22232682]
- [43]. Mestas J, Hughes CCW, Of mice and not men: differences between mouse and human immunology, *Journal of immunology* (Baltimore, Md. : 1950) 172(5) (2004) 2731–2738.
- [44]. O'Hagan DT, Fox CB, New generation adjuvants--from empiricism to rational design, *Vaccine* 33 Suppl 2(S2) (2015) B14–20. [PubMed: 26022561]
- [45]. Mold M, Shardlow E, Exley C, Insight into the cellular fate and toxicity of aluminium adjuvants used in clinically approved human vaccinations, *Scientific Reports* 6(7) (2016) 31578–31578. [PubMed: 27515230]
- [46]. Mangraviti A, Tzeng SY, Kozielski KL, Wang Y, Jin Y, Gullotti D, Pedone M, Buaron N, Liu A, Wilson DR, Hansen SK, Rodriguez FJ, Gao G-D, DiMeco F, Brem H, Olivi A, Tyler B, Green JJ, Polymeric nanoparticles for nonviral gene therapy extend brain tumor survival in vivo, *ACS nano* 9(2) (2015) 1236–49. [PubMed: 25643235]

SIGNIFICANCE STATEMENT

The last several years have brought exciting work exploring biomaterials as delivery vehicles for immunotherapies, vaccines, and gene therapies. However, a gap remains between the striking finding that many biomaterials exhibit intrinsic immunogenic features, and the specific structural properties that drive these responses. The results in the current study indicate PBAEs cause macrophage activation by pathways that are distinct from pathways activated by common vaccine and immunotherapies components, such as toll-like receptor agonists. Thus, the work reveals new mechanistic details that can be exploited in investigating other materials, and to support more rational design of future biomaterial vaccines and immunotherapy carriers.

Figure 1. PBAE-DsiRNA complex assembly and physicochemical characterization. Complexes formed with PBAE (A) and DsiRNA in sodium acetate buffer were characterized for size (B) and zeta potential (C) at varying weight ratios of PBAE:RNA.

Figure 2. Viability of macrophages treated with PBAE.

Cells treated with soluble and complexed PBAE at specified w:w ratios were assessed for viability (DAPI⁻) at 24 hours (A) and 48 hours (B) alongside cells treated with controls using flow cytometry. Representative results from one of three separate experiments are shown.

brackets indicate key statistical relationships (using “n.s.” for not significant) among soluble treatments only.

Figure 4. Morphology after overnight treatment.

Representative micrographs of macrophages (phase contrast, 10X, scale bar: 100 μm) following overnight treatment with soluble PBAE from 0.5 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$ and controls (LPS 100 ng/mL, R848 1 $\mu\text{g/mL}$, polyIC 10 $\mu\text{g/mL}$, and sodium acetate buffer).

Figure 5. IL-6 and IL-10 secretion by macrophages at 24 and 48 hours.

Secretion of IL-6 (A,B) and of IL-10 (C,D) was measured by ELISA from cell supernatants saved during flow cytometry experiments. Representative results from two separate experiments are shown including soluble treatments of PBAE ranging from 0.5–4 μ g/mL (soluble 3 μ g/mL not measured), complexed treatments at 2 and 3 μ g/mL and TLR agonist controls. Samples with no bar indicate the cytokine was not detected.

Figure 6. Activity in mouse TLR reporter cells.

Reporter cells expressing TLR3 (A), TLR7 (B), or TLR9 (C) were screened with soluble or complexed PBAEs and equivalent NC1, buffer, media, and TLR agonist controls. LPS was used as an irrelevant TLR agonist. Representative results from one of three separate experiments are shown. Statistical symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) directly above individual bars indicate significantly higher mean than all soluble controls (buffer, NC1, media).

Figure 7. NF- κ B and IRF activity in reporter cells.

Cell supernatants from reporter cells were screened for NF- κ B-induced secreted embryonic alkaline phosphatase (A,B) and IRF-induced secreted Lucia luciferase reporters (C,D) in absorbance and luminescence assays following 24 and 48 hour incubation with treatments. Cells were treated with soluble PBAE from 0.5 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$ and controls (NC1 in sodium acetate buffer, sodium acetate buffer alone, LPS 100ng/mL, R848 1 $\mu\text{g/mL}$, polyIC 10 $\mu\text{g/mL}$). Representative results from one of two separate experiments are shown. Statistical symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) directly above individual bars indicate significantly higher mean than all soluble controls (buffer, NC1, media).

Table 1

DsiRNA content of dose-matched soluble (S) and complexed (C) PBAE treatments with calculated nitrogen:phosphate (N:P) ratio.

PBAE (µg/mL)	4.0		3.0				2.0				1.0		0.5
Formulation	S	S	C	C	C	C	S	C	C	C	C	S	S
Ratio	-	-	25:1	5:1	2:1	1:1	-	25:1	5:1	2:1	1:1	-	-
DsiRNA (µg/mL)	0	0	0.12	0.6	1.5	3	0	0.08	.4	1	2	0	0
N:P Ratio	-	-	40.8	8.2	3.3	1.6	-	40.8	8.2	3.3	1.6	-	-