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Intrinsic immunogenicity of rapidly-degradable polymers evolves during degradation

James I. Andorko [a], Krystina L. Hess [a], Kevin G. Pineault [a], Christopher M. Jewell [a,b,c,*]

[a] Fischell Department of Bioengineering, University of Maryland, College Park, MD, United States
[b] Department of Microbiology and Immunology, University of Maryland Medical School, Baltimore, MD, United States
[c] Marlene and Stewart Greenebaum Cancer Center, Baltimore, MD, United States

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ABSTRACT

Recent studies reveal many biomaterial vaccine carriers are able to activate immunostimulatory pathways, even in the absence of other immune signals. How the changing properties of polymers during biodegradation impact this intrinsic immunogenicity is not well studied, yet this information could contribute to rational design of degradable vaccine carriers that help direct immune response. We use degradable poly(beta-amino esters) (PBAEs) to explore intrinsic immunogenicity as a function of the degree of polymer degradation and polymer form (e.g., soluble, particles). PBAE particles condensed by electrostatic interaction to mimic a common vaccine approach strongly activate dendritic cells, drive antigen presentation, and enhance T cell proliferation in the presence of antigen. Polymer molecular weight strongly influences these effects, with maximum stimulation at short degradation times – corresponding to high molecular weight – and waning levels as degradation continues. In contrast, free polymer is immunologically inert. In mice, PBAE particles increase the numbers and activation state of cells in lymph nodes. Mechanistic studies reveal that this evolving immunogenicity occurs as the physicochemical properties and concentration of particles change during polymer degradation. This work confirms the immunological profile of degradable, synthetic polymers can evolve over time and creates an opportunity to leverage this feature in new vaccines.

Statement of Significance

Degradable polymers are increasingly important in vaccination, but how the inherent immunogenicity of polymers changes during degradation is poorly understood. Using common rapidly-degradable vaccine carriers, we show that the activation of immune cells – even in the absence of other adjuvants – depends on polymer form (e.g., free, particulate) and the extent of degradation. These changing characteristics alter the physicochemical properties (e.g., charge, size, molecular weight) of polymer particles, driving changes in immunogenicity. Our results are important as many common biomaterials (e.g., PLGA) are now known to exhibit immune activity that alters how vaccines are processed. Thus, the results of this study could contribute to more rational design of biomaterial carriers that also actively direct the properties of responses generated by vaccines.

1. Introduction

Biomaterials have become important components in many emerging vaccine and immunotherapy strategies. These materials provide cargo protection, controlled release of antigens or immune signals, and targeting of immune cells and secondary lymphoid organs such as lymph nodes (LNs) – key tissues that help coordinate immune function [1–3]. Intriguingly, several recent studies demonstrate that poly(lactic-co-glycolic acid) (PLGA), polystyrene, chitosan, and other polymers ubiquitous in the biomedical field exhibit intrinsic immunostimulatory properties, even in the absence of antigens, adjuvants, or other immune signals [1,4–9]. Pattern recognition receptors (PRRs) of the innate immune system have the ability to recognize pathogen-associated and danger-associated patterns through Toll-like receptor (TLR) and

* Corresponding author at: Fischell Department of Bioengineering, 2212 Jeong H. Kim Engineering Building, College Park, MD 20742, United States.
E-mail address: cmjewell@umd.edu (C.M. Jewell).
URL: http://jewell.umd.edu (C.M. Jewell).

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inflammasome pathways (e.g., nucleotide-binding oligomerization domain-like receptors, Rig-like helicases) [10]. These pathways drive production of pro-inflammatory caspases and cytokines that many polymers such as PLGA or polystyrene are able to activate. For example, when dendritic cells (DCs) are treated with PLGA or non-degradable polystyrene particles and a TLR agonist (TLRa), these particles cause an increase in expression of surface activation markers (e.g., CD40, CD80, CD86) [6,7,9]. Many of these important polymers also increase inflammatory cytokines such as IL-1β, a key mediator of the inflammasome that supports early immune responses [6,7,10]. Several studies have investigated the link between these types of inflammatory/immunostimulatory processes and physicochemical polymer properties such as molecular weight (MW) [5,11–13], particle size [8], charge [14], hydrophobicity [15], shape [16], and chemical functionality [17–20]. These studies confirm polymer properties can modulate immune function. Developing a better understanding of these phenomena and how immunogenicity evolves during degradation could support rational design of polymers that serve not only as carriers but also as agents that help direct or tune immune response.

The mechanistic studies above have focused on non-degradable or slowly-degrading polymers such as polystyrene and PLGA, respectively. However, vaccines which allow for tunable, rapid delivery of antigens, adjuvants, or other small molecules offer new potential for modulating the development of specific immune characteristics, for example, by rapidly releasing immune-polarizing drugs during DC activation or T cell expansion. Poly (beta-amino esters) (PBAEs) are cationic, pH-sensitive polymers that degrade over hours to days depending on polymer structure [21,22]. PBAEs have been used in drug delivery, for DNA and RNA delivery, and as vaccine carriers [23–33]. In the latter case, PBAEs have been used for DNA vaccination through condensation of nucleic acids encoding plasmid antigens to promote T cell mediated anti-tumor responses, to drive antibody response, or for siRNA immunotherapy to preferentially target glioblastoma [34–40]. Although PBAEs are becoming increasingly useful in vaccination and immunotherapy, little is known about if and how these materials elicit intrinsic immunostimulatory or inflammatory effects, and how the rapid degradation of PBAEs or other materials changes the intrinsic immunogenic properties in cells or tissues.

We hypothesized that PBAEs would exhibit intrinsic immunostimulatory effects that change as a function of polymer form (i.e., free, particles) and the extent of polymer degradation (MW). To test this idea, a prototypical PBAE, Poly 1, was synthesized from a four-carbon diacrylate monomer and a diamine. The ability of this material to activate DCs and T cells in co-culture and in mice was then assessed (Fig. 1). Free Poly1 did not activate DCs or drive synergistic responses during co-treatment with a TLRa. Since the immune system has evolved to detect particulate materials and pathogens, we tested if the properties of PBAE particles change during degradation and if these effects impact intrinsic immunostimulatory function. In contrast to free polymer, particles formed from intact Poly1 using electrostatic condensation to mimic a common vaccine formulation method exhibited significant intrinsic immunostimulatory function that decreased with the extent of polymer degradation. We discovered that these changes corresponded to changing physicochemical properties including increasingly negative particle charges, increased particle diameter, and decreased particle concentration. Mechanistic studies using particles assembled from Poly1 fragments with distinct MWs confirmed this trend, revealing that Poly1 differentially activates immune cells in culture and mice in a manner that is dependent on both the form of the polymer and the MW (i.e., degree of degradation). These studies confirm the intrinsic immunogenicity of PBAEs and provide insight into how the evolving properties of degradable polymers drive immunogenicity in ways that could support design of new vaccine carriers able to adjuvant or modulate immune function.

2. Materials and methods

2.1. Materials

Monomers for polymer synthesis (1,4-butanediol diacrylate and 4,4′-trimethylenedipiperidine) were purchased from Alfa Aesar (Ward Hill, MA) and Sigma–Aldrich (St. Louis, MO), respectively. Tetrahydrofuran (THF), diethyl ether, sodium acetate (SA) buffer, poly(sodium 4-styrenesulfonate) (SPS), and lipopolysaccharide from Escherichia coli 0111:B4 (LPS) were also purchased from Sigma–Aldrich. THF used in GPC studies was purchased from Macron Fine Chemicals (Center Valley, PA). Agilent LS EASICAL PS-1 polystyrene GPC standards were from Fisher Scientific (Pittsburgh, PA). Deuterated-chloroform ($CDCl_3$) was bought from Cambridge Isotope Laboratories (Tewksbury, MA). RPMI-1640 media was purchased from Lonza (Allendale, NJ). Fetal bovine serum (FBS) was supplied by Corning (Tewksbury, MA). Low MW polyinosinic-polycytidylic acid (PolyIC) was purchased from Invivogen (San Diego, CA). CD11c microbeads were purchased from Miltenyi Biotec (Cambridge, MA). Spleen Dissociation Medium and CD4 negative selection kits were from STEMCELL Technologies (Vancouver, British Columbia, Canada). Fluorescent antibody conjugates were purchased from BD (San Jose, CA) or eBioscience (San Diego, CA). 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) was purchased from Sigma–Aldrich. Evan's blue dye and 40 μm cell strainers were from VWR (Radnor, PA).

2.2. Cell and animals

Female C57BL6 mice (4–8 weeks, stock #000664) and male C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J (2D2) mice (10–12 weeks, stock #006912) were from Jackson Laboratories (Bar Harbor, ME). The 2D2 strain displays $CD4^+$ T cell receptors transgenic for myelin oligodendrocyte glycoprotein, residues 35–55 (MOG_{35-55}). All animals were cared for in compliance with Federal, State, and local guidelines, and using protocols reviewed and approved by the University of Maryland's Institutional Animal Care and Use Committee (IACUC).

2.3. Poly1 synthesis, degradation, and characterization

Poly1 was synthesized via a Michael-type addition reaction as described previously [21,22,24]. Briefly, 9 mmol of 4,4′-trimethyle nedipiperidine was dissolved in anhydrous THF to form a 500 mg mL^{-1} solution. This solution was added to 9 mmol of 1,4-butanediol diacrylate and the reaction was heated to 50 °C and stirred for 16 h. The reaction was cooled to room temperature and the resulting polymer was precipitated in vigorously stirred ice cold diethyl ether. After collecting the polymer and washing with additional diethyl ether, the polymer was lyophilized. A 16 mg mL^{-1} solution of Poly1 was prepared in $CDCl_3$ and ^1H NMR was used to confirm the structure. Poly1 was dissolved in THF at 2.5 mg mL^{-1} and a THF-based gel permeation chromatography (GPC) system (Waters) was used to determine polymer MW compared to polystyrene standards. For degradation studies, Poly1 was placed in either pH 7 buffer (1× PBS) or pH 5 buffer (100 mM SA) and incubated at 37 °C for increasing intervals to form polymer fragments. Following incubation, the degraded samples were lyophilized and MW (weight average) was determined by GPC.

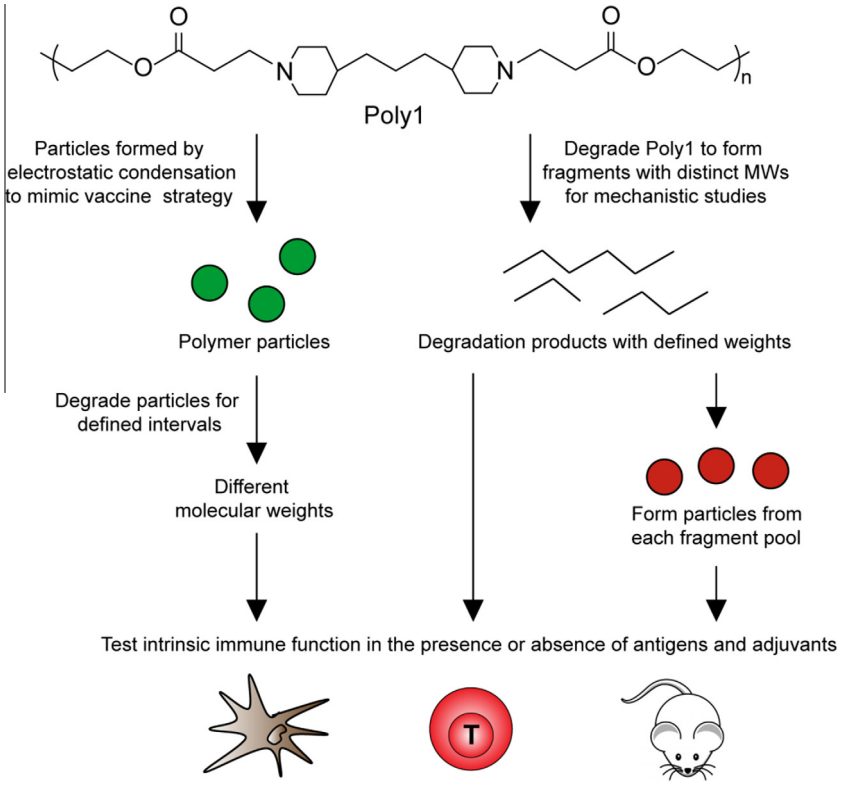

Fig. 1. Schematic depicting the approach to investigate the intrinsic immunogenicity of Poly1, a degradable, cationic PBAE. DCs, T cells, and mice are treated with free Poly1 or one of two Poly1 particle formulations: (i) particles formed from intact Poly1 then degraded to mimic a common vaccine formulation strategy, or (ii) pools of Poly1 degradation fragments with distinct MWs formed into particles to mechanistically study the link between PBAE properties (e.g., MW, physical form) and DC activation, antigen presentation, and T cell function.

2.4. Poly1 particle formation and characterization

Poly1 particles were assembled from either intact polymer then degraded, or assembled from polymer fragments with distinct MWs formed by degradation prior to particle assembly. For the former studies, intact Poly1 was used to prepare particles via electrostatic condensation by mixing 5 mM Poly1 and 20 mM SPS at a SPS:Poly1 w/w ratio of 1:1.6. These particles were then incubated at 37 °C for specific intervals to determine how degradation of particles influences the intrinsic immune activity. For mechanistic studies involving pre-degraded polymer, Poly1 was degraded in pH 5 buffer for specific intervals to form fractions with distinct MW fragments of Poly1, then mixed with SPS as above to prepare particles. For each particle preparation, 400 μg of 5 mM Poly1 was used to form particles with SPS in pH 5 water while maintaining a fixed overall volume. Thus the amount of polymer (Poly1, SPS) was constant in either case, irrespective of whether particles were formed from intact Poly1 then degraded, or assembled from Poly1 fragments. GPC, as above, was used to quantify the extent of degradation for Poly1 particles formed then degraded. Laser diffraction (Horiba LA950, Edison, NJ) was used to determine the diameter of the particles. Zeta potential was determined using a Malvern Zetasizer Nano ZS90 (Westborough, MA).

2.5. DC activation, antigen presentation, and flow cytometry

Primary $CD11c^+$ DCs were isolated from spleens of C57BL6 mice via positive selection using the recommended protocols for Spleen Dissociation Medium (STEMCELL) and CD11c Microbeads (Miltenyi). Isolated DCs were plated at 100,000 cells per well in a 96 well plate and cultured at 37 °C, 5% CO_2. DCs were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1× non-essential amino acids, 10 mM HEPES buffer, 1× penicillin and streptomycin, and 55 μM β-mercaptoethanol. For DC activation studies, DCs were treated with either buffer ("vehicle"), 10 μg mL^{-1} of TLR3a (PolyIC), 1 μg mL^{-1} of TLR4a (LPS), 400 ng of Poly1 in free form or in one of two particle formulations: (i) particles assembled from intact Poly1 then degraded, or (ii) particles assembled from each MW fraction of degraded polymer. A subset of these formulations was also studied in combination with the TLRa treatments, as indicated. For all DC studies, 48 h after treatment, cells were stained for viability (DAPI) and for surface activation markers (i.e., CD40, CD80, CD86, and Major Histocompatibility Complex II (MHCII)) using fluorescent antibodies conjugates. Cells were examined by flow cytometry (BD CantoII, San Jose, CA) and data were analyzed using FlowJo v. 10 (TreeStar, Ashland, OR). For antigen presentation assays, DCs were co-incubated with 1 μg of a model antigen (SIINFEKL) in soluble form and each of the polymer formulations indicated and hypothesized to exhibit an adjuvant effect. After 48 h, cells were stained with an antibody specific to SIINFEKL presented via MHCI ($H\text{-}2K^b$) then analyzed by flow cytometry to determine the percentage of DCs processing and presenting SIINFEKL in MHCI.

2.6. DC/T cell co-culture and T cell proliferation

For studies involving DC and T cell co-cultures, primary DCs ($CD11c^+$) were isolated from C57BL6 mice as above, and then treated with Poly1 in free or particle form. A sub-optimal dose (0.05 μg in 10 μL) of a model antigen (MOG_{35-55}) in soluble form was added to all wells to serve as the cognate antigen for transgenic 2D2 T cells. After 48 h in culture, $CD4^+$ T cells were isolated from the spleens of 2D2 mice using the manufacturer's recommended protocol (STEMCELL). Following isolation, T cells were labeled with CFSE by adding 50 μM of CFSE (in media) per mL of cells, incubated for 5 min at room temperature, and then washed twice with 10

volumes of media. 300,000 CFSE-labeled $CD4^+$ 2D2 T cells were added to each well containing DCs, then incubated for 72 h with 50% media replacement every 24 h. After incubation, cells were collected, and stained with DAPI and antibodies specific to the T cell surface markers (i.e., CD3e, CD4). Flow cytometry was used to determine the extent of T cell proliferation via CFSE dilution, with cells gated beyond the second generation selected as a criterion for proliferated cells.

2.7. i.LN injection of Poly1 into inguinal LNs of mice

For *in vivo* studies, Poly1 that degraded for 24 h was injected into the inguinal LNs of mice in free or particle form. In a typical study, a small region of fur was removed from the lateral hind quarter of C57BL6 mice by shaving the area and applying a mild depilatory. The mice were then injected subcutaneously with a tracer dye (Evan's Blue) on each side of the tail base as previously reported [41,42]. After allowing time for the tracer dye to drain to the inguinal LNs for visualization, a 31G insulin needle was used to inject 10 μL (6.5 μg) of free Poly1, Poly1 particles, or a sham consisting of buffer into each inguinal LN.

2.8. In vivo DC activation, LN cell phenotyping, and lymphadenopathy

24 h after injection, mice were euthanized and the inguinal LNs were removed and placed in PBS. A single cell suspension was prepared by passing each LN through a 40 μm strainer with PBS and the total cell number was enumerated using an automated cell counter (NanoEnTek, Pleasanton, CA). Cells were then stained in a similar manner to co-culture studies and analyzed by flow cytometry for viability ($DAPI^-$), phenotype (DC, $CD11c^+$; B cell, $B220^+$; T cell, $CD3^+$), and DC activation markers (i.e., CD40, CD80, CD86, MHCII).

2.9. Statistical analysis

One way ANOVA with a Tukey post-test was performed using Graphpad Prism (version 6.02) for statistical testing. A p-value of 0.05 or less was considered significant.

3. Results

3.1. Poly1 degrades to form low MW fragments

Poly1 was synthesized and the reaction product was confirmed via NMR after 16 h of synthesis (Fig. 2A). Poly1, with a MW of 3.8 kDa (Fig. 2B), was then dissolved in pH 5 buffer. The MW of Poly1 decreased to 39.9% (~1.5 kDA) of the starting MW over 24 h and to 8.4% (0.3 kDa) of the starting MW over one week (Fig. 2B and C). This degradation rate corresponded to a half-life of ~17.7 h and as previously reported [21], Poly1 degrades to form byproducts of 1,4-butanediol and bis(β-amino acid). The intrinsic immunogenicity as polymers degrade to fragments was then studied in culture and in mice using Poly1 in different forms (i.e., free, particles) and at different extents of degradation (i.e., MWs).

3.2. Poly1 in free form does not activate DCs

DCs were incubated with Poly1 degradation fragments that were 100%, 88%, 40%, 20%, or 8% of the starting MW. These fragments were used to treat primary DCs in the absence of antigen or adjuvants. Following treatment, common DC activation markers were investigated including: (i) MHCII, a molecule responsible for presenting antigen to T cells, (ii) CD80 (B7-1) and CD86 (B7-2), costimulatory markers co-expressed with MHC complexes for proper T cell activation, and (iii) CD40, a membrane protein expressed on activated DCs during antigen-specific immunity [43–46]. Compared with control cells treated with buffer (Fig. 3, "vehicle"), free Poly1 (Fig. 3, blue) did not increase characteristic DC activation markers including CD40 (Fig. 3A), CD80 (Fig. 3B), CD86 (Fig. 3C), and MHCII (Fig. 3D). To test if free Poly1 mediated a synergistic effect on DC activation in the presence of other adjuvants, DCs were co-cultured with Poly1 fragments and a TLR3a (PolyIC, Fig. 3, red) or a TLR4a (LPS, Fig. 3, green). DCs treated with PolyIC or LPS were strongly activated, but DCs treated with both Poly1 and either TLRa did not exhibit any further increases in activation. These data indicate that Poly1 in free form does not exhibit strong intrinsic immunogenicity or cause synergistic effects in the presence of other adjuvants (i.e., TLR3a, TLR4a).

3.3. Particles assembled from intact Poly1 exhibit the greatest levels of intrinsic immunogenicity at early stages of degradation

We hypothesized that the form of polymer in solution may impact the intrinsic immune characteristics of Poly1 by altering the ability of APCs to detect, internalize, and process Poly1. To test this idea in a well-controlled manner, particles were formed by electrostatic condensation of Poly1 with a stable polyanion (SPS). This approach mimics a common particle synthesis strategy used in DNA vaccination whereby antigen-encoding plasmid is condensed by PBAEs or other cationic polymers or lipids. These particles were then incubated in buffer at 37 °C. GPC results confirmed that over time, Poly1 in the particles continued to degrade, leading to a reduction in MW and a lower particle concentration that resulted in increased optical transmittance (Fig. 4). During this process, particle size increased, while zeta potential – which was initially positive (~15 mV) – decreased (Fig. 5A and B). Further, the number of particles decreased to undetectable levels as degradation progressed (Figs. 4C and 5A). Particle size measurements (volume basis, Fig. S1A) and image analysis (Fig. S1C) at each stage of degradation revealed that the decreased particle concentration was due not only to degradation, but also from aggregation of individual particles into larger, but fewer, agglomerates. After confirming SPS had no effect on cell viability (Fig. S2), we screened the immunogenicity of the Poly1 particles by treating DCs with particles at each stage of degradation. Poly1 particles at early stages of degradation strongly activated DCs, then waned as a function of increasing particle degradation time and decreasing MW (Fig. 5C–F). These effects were most evident in CD86 and CD80 expression – markers associated with co-stimulation. Thus, particles formed from Poly1 exhibit intrinsic immunogenicity, and these effects vary as a function of polymer degradation which alters the physicochemical properties of these particles. Building on this discovery, we designed a series of experiments to examine how the extent of polymer degradation influences particle properties and immune function in cells and mice.

3.4. Immunogenicity of particles assembled from Poly1 fragments depends on the extent of degradation

To more directly isolate the role of MW in defining the intrinsic immunogenicity of PBAEs, we used Poly1 fragments with distinct MWs as in Fig. 2 to form particles through condensation with SPS. As observed with particles degraded after formation (Fig. 4C), the concentration of Poly1 particles formed from distinct MW fragments generally decreased with degradation time while transmittance increased. Interestingly, a slight increase in particle concentration was observed at 4 h (Fig. 6A), and also observed by microscopy (Fig. S1D). Depending on the degree of degradation, particles formed using each pool of Poly1 fragments exhibited sizes of 1.62 μm for intact polymer, increasing to 9.96 μm when

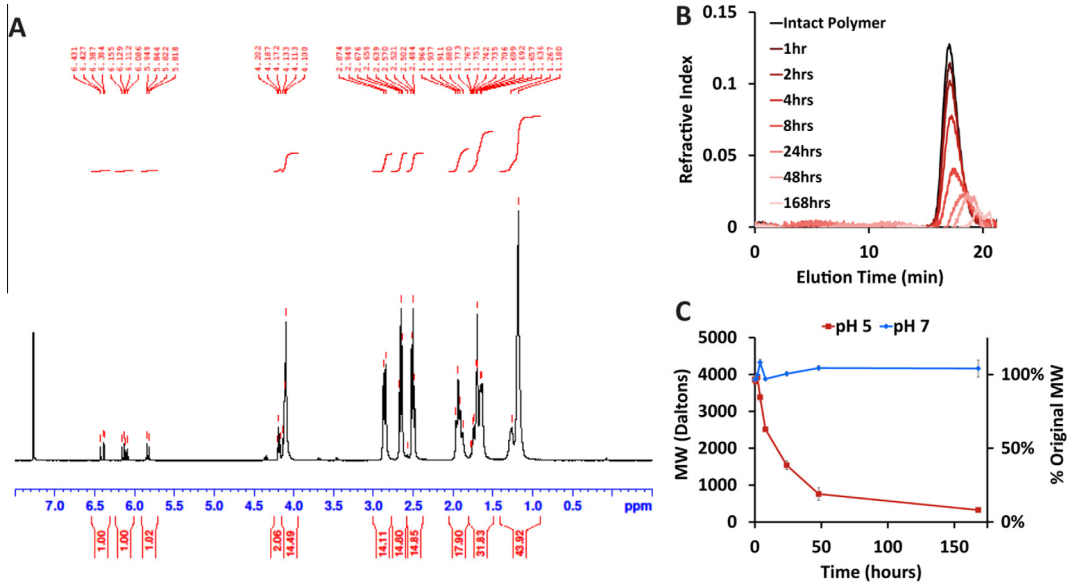

Fig. 2. Poly1 synthesis, characterization, and degradation to low MW polymer chains over one week in buffer. (A) ^{1}H NMR shifts of Poly1 integrated with respect to deuterated chloroform including chemical shift definitions. (B) GPC chromatograph depicting the change in MW of Poly1 after degradation in pH 5 buffer (100 mM SA). Increased elution time correlates to lower MW fragments. (C) Poly1 MW changes after incubation in pH 5 and pH 7 (1× PBS) buffers. Data are relative to polystyrene standards and pooled from three independent experiments for each incubation time. Error bars represent SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

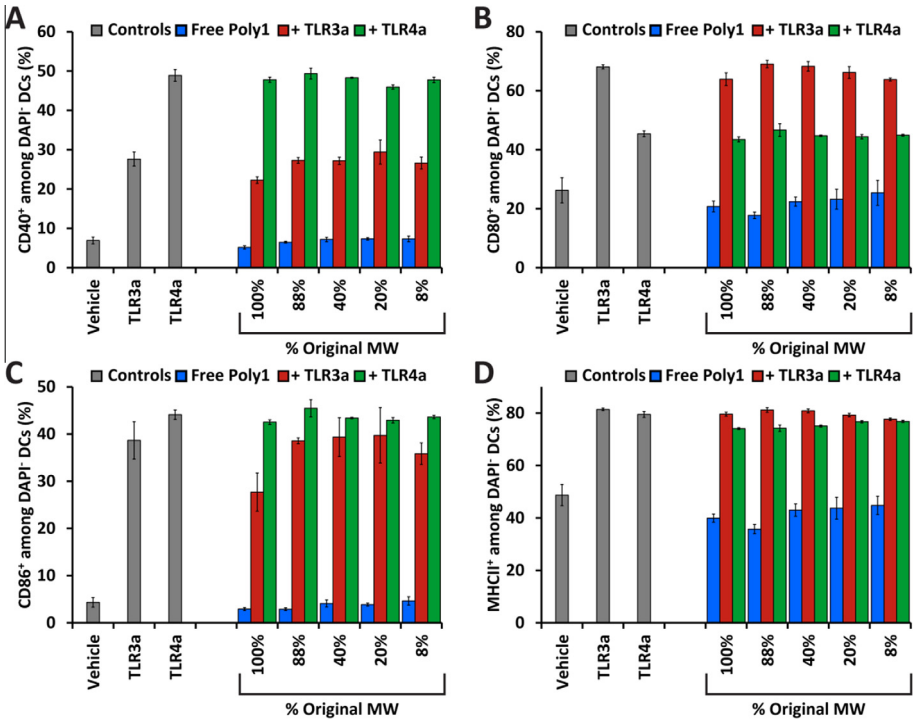

Fig. 3. Free Poly1 does not activate DCs. Expression levels of (A) CD40, (B) CD80, (C) CD86, or (D) MHCII on primary CD11c^{+} DCs treated with free Poly1 degradation fragments of varying MW. Cells were incubated for 48 h with the indicated Poly1 fragments in the absence (blue) or presence of a TLR3a (PolyIC, red) or TLR4a (LPS, green), respectively. Expression levels are indicated among DAPI^{-} cells and are representative of three similar experiments with samples conducted in triplicate with error bars depicting SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

particles were formed from fragments collected after a one week degradation period (Fig. 6B). The zeta potential of particles, which was positive (~15 mV) at early stages, became highly negative as particles were formed with Poly1 fragments at greater extends of degradation; particles formed with Poly1 fragments degraded for one week exhibit values of −9 mV (Fig. 6C). In general, as the degree of degradation increased, particles exhibited more negative surface charges, and by the end of the study, a greatly increased diameter. Compared to the aggregation observed in particles formed prior to degradation (Fig. S1A and C), particles formed from pre-degraded Poly1 remained largely free of aggregation until mostly degraded by 168 h. These results were indicated by particle size analysis (Fig. S1B) and inspection of microscopy images (Fig. S1D). We next tested the ability of these particles to activate

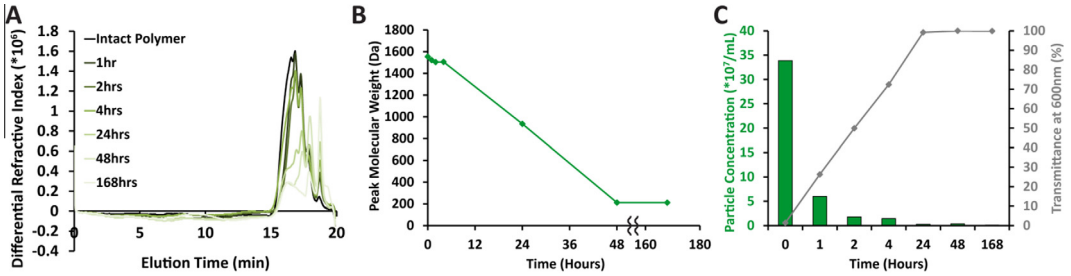

Fig. 4. Poly 1 particles degraded for specific intervals following formation exhibit a rapid drop in MW that decreased particle concentration. (A) GPC chromatograph depicting the change in MW of Poly1 particles after degradation in pH 5 buffer (100 mM SA). Increased elution time correlates to lower MW fragments. (B) The peak corresponding to the MW distribution of Poly1 changes as particles degrade in pH 5 buffer. (C) Particle concentration and transmittance of particles formed by condensing intact Poly1 prior to degradation changes with the extent of degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

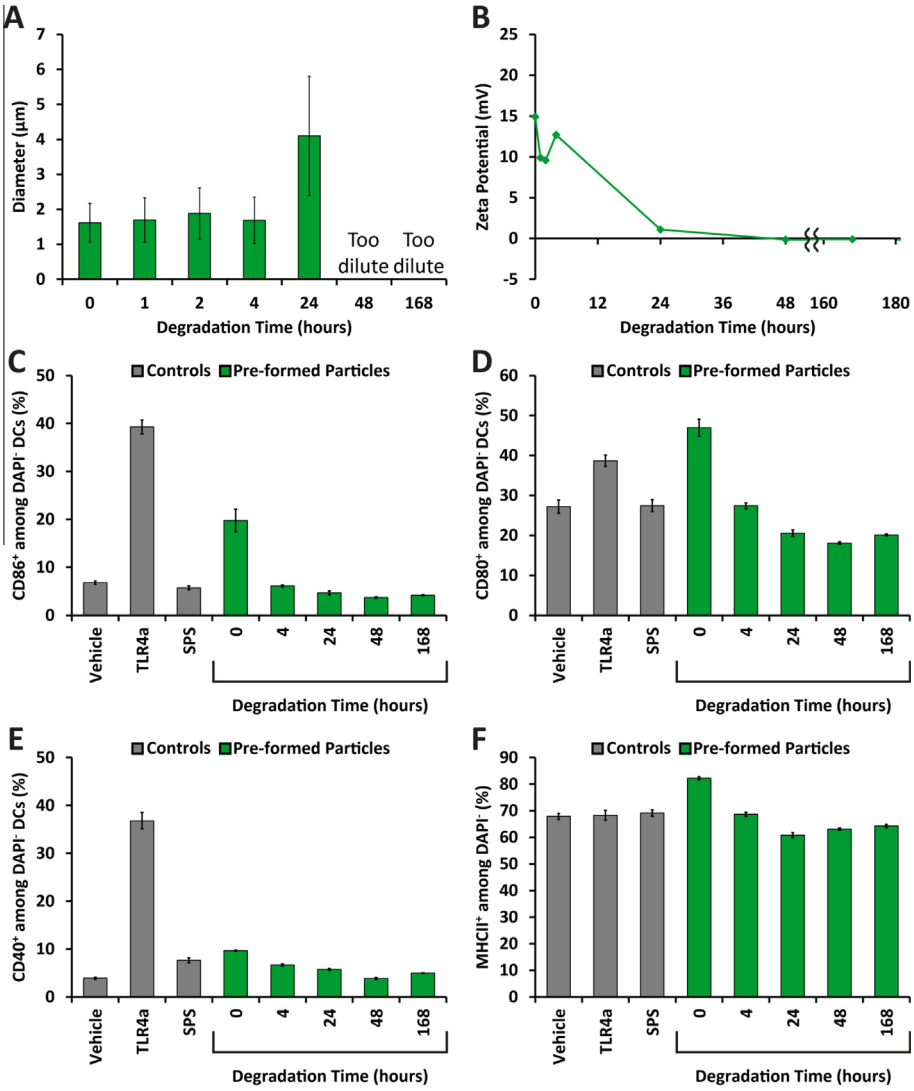

Fig. 5. Pre-formed particles exhibit intrinsic characteristics that activate DCs during early stages of particle degradation. (A) Particle size and (B) zeta potential shift as a function of degradation time. "Too dilute" designates formulations for which size measurements could not be reliably obtained due to insufficient particle concentration at long degradation times. Expression of (C) CD86, (D) CD80, (E) CD40, or (F) MHCII after treatment of $CD11c^+$ DCs with 400 ng of Poly1 particles at varying extents of degradation. Data correspond to samples prepared in triplicate with error bars representing SEM. For panel B, error bars are smaller than the data markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DCs. Poly1 particles exhibited significant levels of DC activation when prepared using intact Poly1 or Poly1 fragments from short degradation times (i.e., high MW) (Fig. 6D–G). These effects then decreased as a function of polymer degradation time, with near-baseline activation levels by the end of the study. Interestingly, CD80 and CD86 levels were elevated with Poly1 fragments degraded to 40% of the original MW, an observation that correlated with a transiently-elevated particle concentration at this point.

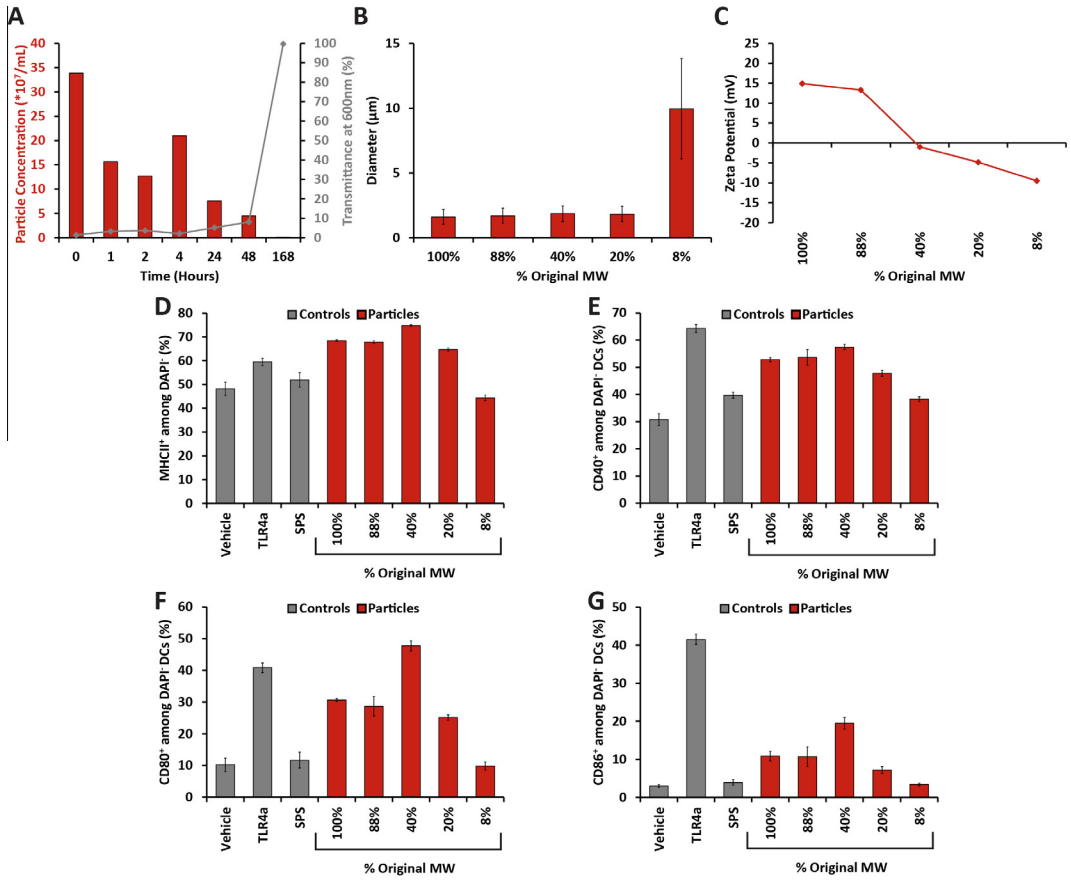

Fig. 6. Poly1 particles formed with pre-degraded Poly1 fragments induce MW-dependent activation of DCs. (A) Particle concentration and transmittance of particles formed by condensing Poly1 fragments generated by degradation in buffer prior to condensation. (B) Particle size and (C) zeta potential of Poly1 particles alters with the extent of degradation. Expression levels of (D) MHCII, (E) CD40, (F) CD80, or (G) CD86 following treatment of primary $CD11c^+$ DCs with 400 ng of Poly1 particles at different extents of degradation. Vehicle and TLR4a indicate cells treated with buffer or LPS, respectively. Analysis was conducted 48 h after treatment. Expression levels are indicated among $DAPI^-$ cells (i.e., live cells). Data are representative of three similar experiments with samples prepared in triplicate. Error bars represent SEM. For panel C, error bars are smaller than the data markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Poly1 particles increase antigen presentation through MHCI and MHCII, and drive T cell expansion

To test if Poly1-induced DC activation impacts antigen presentation, DCs were treated with a model antigen (SIINFEKL) and either free Poly1 or particles formed from each MW pool of Poly1 fragments. SIINFEKL presented by these cells in MHCI ($H\text{-}2K^b$) was then quantified by flow cytometry. While free Poly1 (Fig. 7A, blue) did not cause a significant increase in antigen presentation compared to controls (Fig. 7A, gray), DCs treated with Poly1 particles (Fig. 7A, red) caused a dramatic increase in SIINFEKL presentation. In agreement with DC activation data (Fig. 6D–G), presentation levels depended on the extent of degradation, with the highest levels associated with Poly1 degraded for short times (i.e., higher MWs) (Fig. 7A). These results indicate that Poly1 particles stimulate DCs in a manner that enhances the ability of DCs to effectively present antigen via the MHCI pathway – an arm of adaptive immunity important in response to viral infection.

To determine if DC activation and antigen presentation induced by Poly1 particles drives T cell expansion, $CD11c^+$ DCs from C57BL6 mice were co-cultured with $CD4^+$ transgenic T cells from 2D2 mice. T cells from 2D2 mice display T cell receptors specific for a well-defined cognate antigen (MOG_{35-55}). In these studies, DCs were cultured for 48 h with MOG_{35-55} and either free Poly1, Poly1 particles formed with pre-degraded Poly1, LPS, or buffer. Freshly isolated $CD4^+$ 2D2 T cells labeled with CFSE were then added to the culture for 72 h. Fig. 7B depicts representative flow cytometry traces of CFSE dilutions (among $DAPI^-/CD3^+/CD4^+$ cells) for each treatment group. Samples treated with vehicle and MOG_{35-55} or positive controls (i.e., LPS with MOG_{35-55}), caused 8.26% (Fig. 7B, light gray) and 36.3% (Fig. 7B, dark gray) of T cells to proliferate beyond the second generation (Fig. 7B, left gates), respectively. Consistent with DC activation and antigen presentation studies (Figs. 6 and 7A), cultures treated with free Poly1 (Fig. 7B, blue) did not exhibit differences in T cell proliferation compared to the vehicle control (Fig. 7B, gray). Intriguingly, cells treated with Poly1 particles (Fig. 7B, red) induced strong T cell proliferation, resulting in three distinct generations beyond the second generation. This expansion was comparable to that observed in co-cultures treated with a strong TLR3a or TLR4a (i.e., PolyIC, LPS, respectively) and much greater than the negligible expansion observed in vehicle-treated cultures (Fig. 7B–D). Compared with free Poly1, Poly1 particles caused significantly more proliferation as reflected in both MFI analysis (Fig. 7C) and as a percentage of proliferated cells for each formulation (Fig. 7D). The effect of Poly1 particles on proliferation was also weakly influenced by the extent of degradation (i.e., MW), with proliferation generally decreasing at lower MWs (Fig. 7C and D). However, the statistical power of these trends were weak. Thus, Poly1 particles induce DC activation and antigen presentation that drives antigen-specific T cell expansion in a manner that is at least partially dependent on the extent of polymer degradation.

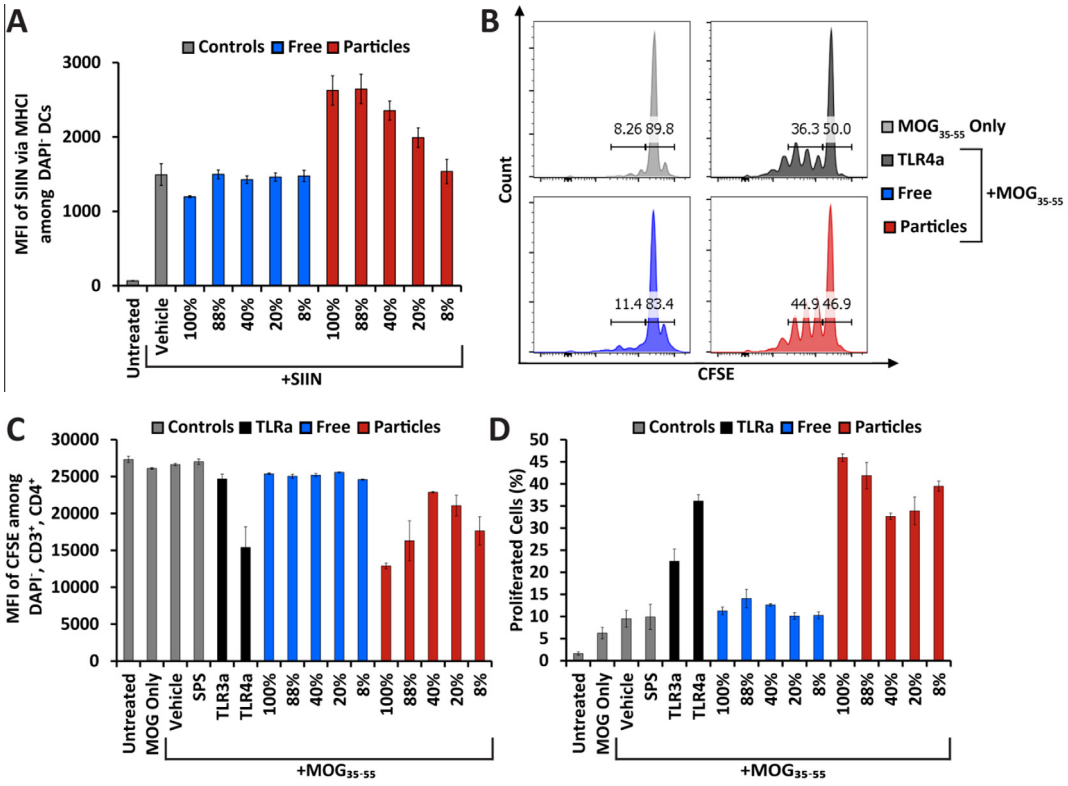

Fig. 7. Poly1 particles increase MHCI-mediated antigen presentation and antigen-specific T cell proliferation. (A) MFI of signal pertaining to SIINFEKL presented in MHCI following treatment of DCs with no SIINFEKL ("Untreated", gray), buffer with SIINFEKL ("SIIN Only", gray), free Poly1 with SIINFEKL (blue), or Poly1 particles with SIINFEKL (red) and gated under $DAPI^-$ cells. 48 h after treatment of DCs as in (A) but using 0.25 μg mL^{-1} of MOG_{35-55}, $CD4^+$ cells isolated from 2D2 mice were added to the wells and incubated for 72 h. (B) Representative flow cytometry traces showing CFSE dilutions after treatment with MOG_{35-55} and either buffer ("MOG Only", gray), TLR4a LPS ("TLR4", black), free Poly1 ("Free", blue), or Poly1 particles formed ("Particles", red). (C) MFI of CFSE signal among $DAPI^-/CD3^+/CD4^+$ cells following treatments as in (B). (D) Percentage of $DAPI^-/CD3^+/CD4^+$ cells proliferated beyond the 2nd generation following treatment as in (B) as determined by CFSE dilution. Samples were prepared in triplicate and errors bars represent SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Poly1 particles activate DCs and expand immune cells in mice

To study the effect of Poly1 on secondary lymphoid organs and local immune function *in vivo*, we used a technique we recently established for direct, intra-lymphatic injection to LNs [41,42]. In these studies, vehicle (i.e., buffer), free Poly1, or Poly1 particles were introduced to the inguinal LNs of mice. After 24 h, mice were euthanized and LNs were collected. Following treatment, the percentage of $CD11c^+$ DCs found in the LNs treated with vehicle, free Poly1, or Poly1 particles (Fig. 8A) did not significantly change, regardless of treatment. Next, we assessed the impact of Poly1 treatment on the activation of LN-resident cells by measuring expression of CD40 (Fig. 8B), CD80 (Fig. 8C), CD86 (Fig. 8D), and MHCII (Fig. 8E). Treatment with free Poly1 (Fig. 8, blue) did not alter activation compared with the vehicle injection control (Fig. 8, gray). In contrast, Poly1 particles (Fig. 8, red) caused a significant increase in the percentage of cells expressing each marker compared to vehicle or to free Poly1 treatments. In line with the results of the DC activation studies, treatment with Poly1 particles (Fig. 8F, red) also increased the total number of cells compared to treatments with vehicle (Fig. 8F, gray) or free Poly1 (Fig. 8F, blue). No significant differences were observed in the relative levels of B and T lymphocytes (Fig. 8G and H) between each group, although treatment with Poly1 particles appeared to cause a modest increase in T cells and a corresponding decrease in B cells. These results indicate that Poly1 exhibits intrinsic immunogenicity both in primary immune cells and in animals.

4. Discussion

In vaccination and immunotherapy, biomaterials offer the ability to control the delivery kinetics of immune signals; however, how the intrinsic immunogenicity of synthetic carriers change as these materials degrade is an area that is not well studied. Several recent studies demonstrate the intrinsic effects of polymer chemistry and physicochemical properties. For example, the Rotello group investigated the effect of specific chemical groups on inflammatory cytokine production using well-controlled chemistry to display these moieties on stable, gold nanoparticles [15]. Here, we have carried out mechanistic studies to test if the changing properties of polymer particles during degradation alters DC activation, processing and presentation of antigen through MHC-I and MHC-II pathways, expansion of antigen-specific T cells, and immunogenicity in mice. To explore these possibilities, we used PBAEs as a rapidly-degradable carrier platform. Since PBAEs are commonly used to condense nucleic acids for gene therapy or DNA vaccination, we formed particles by electrostatic condensation to mimic this polymer-enable vaccination strategy [34–40]. This approach also eliminated stabilizers, surfactants, or other complex compositions required for nanoparticle and microparticle vaccines synthesized by methods such as emulsion/phase inversion. These additional components could hinder isolation of the immunogenic effects of PBAEs and the change in these characteristics during degradation.

Our primary findings in this study are (i) that PBAEs exhibit intrinsic immunogenicity, and (ii) that the extent of this activity

depends both on the form and extent of degradation of Poly1. Past studies with low MW fragments of polymers such as hyaluronic acid indicate that these materials - which were 4–14 oligosaccharides in size (1500–5300 Da) - induce DC activation, cytokine secretion, and T cell proliferation, while high MW hyaluronic acid (80,000–1,000,000 Da) does not [11,12]. In contrast, our studies with free PBAEs reveal that free Poly1 does not activate DCs (Fig. 3). This observation may result from the inability of DCs to efficiently internalize the soluble/relatively low MWs that both intact and degrading Poly1 exhibit. Although DCs use pinocytosis to internalize smaller antigens, DCs are able to sample larger, particulate antigens more efficiently through phagocytosis [47].

Unlike free Poly1, particles formed from intact Poly1 were immunogenic, exhibiting the greatest activity at early stages of degradation, with diminishing intrinsic function at longer times. Poly1 MW became significantly lower at these longer intervals, while the charge became negative, particle size became much larger, and particle concentration decreased (Figs. 4 and 5A and B). Thus, a likely explanation for the decreasing immunogenicity in Fig. 5C–F observed with increased degradation time is (i) the reduced number of particles to interact with immune cells, (ii) the negative surface charge that could hinder association with negatively charged cell membranes [48], and (iii) large particle sizes that limits the efficiency of endocytosis.

Our findings that Poly1 particles formed from intact Poly1 exhibit changing immunogenicity as Poly1 degrades motivated mechanistic studies to investigate more directly how Poly1 fragments alter the properties – and resulting immunogenicity – of particles. Thus we prepared particles from pools of Poly1 fragments with distinct MW ranges. As with our studies involving particles degraded after formation from intact Poly1 (Fig. 5), we discovered that particle size increased inversely with the MW of the Poly1 fragments used for assembly, and zeta potential became increasingly more negative with increased degradation time (Fig. 6). In general, the trends in DC activation were also similar (Fig. 6D–G), with greater activation at lower extents of degradation where particle size was low (~2 μm) and zeta potential was less negative. When Poly1 particles were formed from smaller MW fragments to mimic longer degradation times, particles became larger (Fig. 6B) and surface charge shifted from positive to neutral or slightly negative (Fig. 6C). However, we also observed a transient elevation in activation with particles formed from fragments 40% of the starting MW, though the statistical power of this trend was weak. Our observation of this effect underscores an important role for particle concentration, as the properties of particles formed with these fragments resulted in an increased concentration of particles (Figs. 6A and S2D) for the same polymer mass used for the other fragment pools. Together, these data suggest several conclusions. First, activation is high when particles are at an easily-internalized size (e.g., one to several microns), but before exhibiting a strong negative surface charge that could hinder electrostatic association with negatively charged cell membranes. As degradation continues to greater extents (e.g., 8% of starting MW), particle diameters reach sizes that may be too large to internalize and exhibit negative surfaces. Second, since the Poly1 dose was fixed, particle concentration changes both because polymer is degrading and due to aggregation that results in larger, but fewer, particles. These effects result in particle concentration generally decreasing with time. The transient increase in concentration – and corresponding increase in DC activation – observed with the 40% fragments could result from dispersion of small aggregates (e.g., dimers or trimers) that may form during initial condensation, but that are separated as the particles begin to degrade. However, additional studies are needed to confirm this possibility. In support of the types of

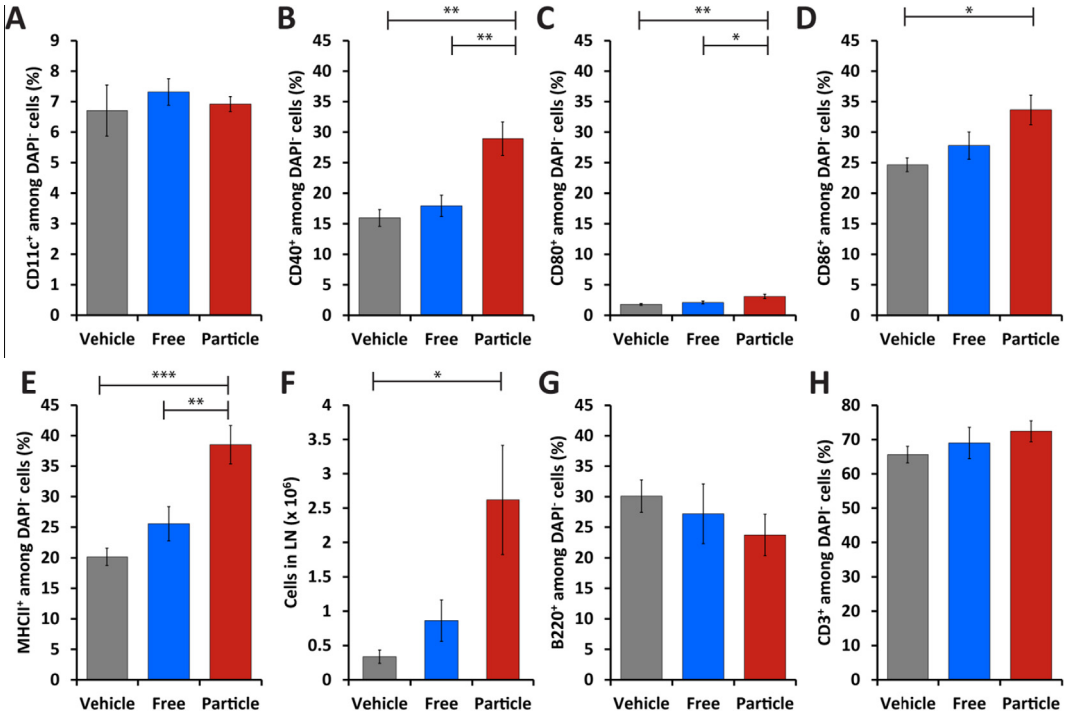

Fig. 8. Poly1 particles induce DC activation and lymphadenopathy without altering cell composition after *i.LN* injection. (A) Percentage of $CD11c^+$ DCs in LN and expression levels of DC activation markers (B) CD40, (C) CD80, (D) CD86, and (E) MHCII following treatment of C57BL6 mice with buffer alone ("Vehicle", gray), 24 h degraded (40% original of MW) free Poly1 ("Free", blue), or Poly1 particles ("Particle", red). (F) Lymphadenopathy as determined by total cell number and percentage of B cells (G) and T cells (H) in the LN following treatment as in (A–E). Data are representative of measurements of at least four LNs with error bars representing SEM. ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interactions discussed in this section, Bishop et al. similarly observed that directly synthesizing PBAEs with different MWs impacts the size and zeta potential of electrostatically-condensed PBAE particles, though this study did not investigate the immunogenicity of the particles [49]. Together, the two approaches we used to characterize the impact of MW on the changing immunogenicity of Poly1 reveal that these changes are a function, at least in part, of changes in particle charge, size, and concentration that result during degradation.

Another interesting result was observed in studying the impact of Poly1 particle treatment on the presentation of antigen through MHCI and MHCII. The MHCI pathway is important in promoting cell-mediated (i.e., $CD8^+$ T cells) responses to destroy cells infected with intracellular pathogens (i.e., viruses) and in new therapeutics such as cancer vaccination and immunotherapy. Our studies reveal an increase in SIINFEKL antigen presentation at low extents of degradation (Fig. 7A). Using T cells from transgenic mice, we also discovered that, in general, Poly1 particles cause an adjuvant effect to promote antigen-specific proliferation through the MHCII pathway at levels that are comparable to those observed in mice treated with LPS and the cognate antigen (Fig. 7B–D). Further, proliferation was not observed when only the antigen was present. Proliferation was only a weak function of MW, though this may be expected due to the exponential nature of T cell expansion and the complexity of the signals playing a role in engaging the T cell receptor (e.g., DC signals, cytokines/soluble factors, cell–cell contacts). These co-culture studies demonstrate that Poly1 particles serve as adjuvants by activating DCs to present antigen through both the MHCI and MHCII pathways, as well as enhancing the co-stimulatory molecules needed to expand antigen-specific T cells. These data also highlight the ability of Poly1 – even in the absence of any other immune cues – to drive functional responses in a manner that is dependent on the form and degradation state of the polymer.

In mice, we discovered that Poly1 alters the composition and function of LNs. Local injection in these studies allowed careful control over the composition and dose of the materials in LNs, revealing that Poly1 is immunogenic *in vivo* and that the level of this activity depends on the material properties (Fig. 8). Treatment with Poly1 particles led to an increase in the number of cells in LNs and activated DCs, while the effects of free Poly1 on LN-resident cells were more modest. Together, these observations suggest that the intrinsic immunogenicity of Poly1 promotes a general (innate) stimulatory effect, with only a weak polarization of the T or B cell compartment. However, these analyses were carried out after 24 h; at later time points and in future studies when antigen and adjuvant are present, antigen-specific effects may be observed. Supporting this possibility, a small biasing toward the T cell compartment was observed in LNs after treatment with Poly1 particles, slightly increasing the frequency of T cells (Fig. 8H) and slightly decreasing the frequency of B cells (Fig. 8G).

The "danger model" describes the evolution of immune pathways that function by detecting pathogens through broad structural features that are uncommon in animals and humans [50]. Some of these characteristics include materials in a particulate form (e.g., virion particles, bacterial cells), short hydrophobic fragments, and polymers or other repetitive structures such as the polysaccharides that commonly comprise bacterial membranes. In our studies, when Poly1 particles were at an optimal size for internalization and positive or neutral, activation was greatest. These effects correlated to other functions such as antigen presentation and proliferation. However at low MWs, activation diminished. Thus, in line with the danger model, our results suggest that broad physicochemical features (e.g., size, charge) – as well as direct effects from changing MW and number of repeat units – account for the evolving immunostimulatory properties of Poly1.

5. Conclusion

Previous studies have confirmed the intrinsic immunogenicity of several biomaterials important in the delivery of drugs, vaccines, and immunotherapies. However, these studies have not focused on how this activity may evolve as materials degrade, and have not been carried out using rapidly-degradable polymer classes such as PBAEs. Here, we have demonstrated that PBAEs, an important polymer class in recent drug delivery and vaccine studies, exhibit strong intrinsic immune effects in a particle form, but not in a free form. Our results also indicate that these effects depend on the extent of polymer degradation, and at least in part, occur because the degrading polymer impacts particle properties such as size, charge, and concentration. Future studies involving treatment of mice with PBAEs at differing extents or rates of degradation will reveal how these materials impact LN structure and function with respect to polymer properties. This approach could also provide new knowledge of how the intrinsic immunogenicity observed here or with other polymers translates to polarization of functional immune outcomes when antigens or other adjuvants are present. Ultimately, this knowledge could contribute to the design of polymers that allow better control over the types, durations, and magnitudes of responses generated with new vaccines or immunotherapies.

Disclosure

The authors do not have any conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.12.026.

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