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Sterile inflammation induced by Carbopol elicits robust adaptive immune responses in the absence of pathogen-associated molecular patterns

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ABSTRACT

Carbopol is a polyanionic carbomer used in man for topical application and drug delivery purposes. However parenteral administration of Carbopol in animal models results in systemic adjuvant activity including strong pro-inflammatory type-1 T-cell (Th1) polarization. Here we investigated potential pathways of immune activation by Carbopol by comparison with other well-characterized adjuvants. Carbopol administration triggered rapid and robust leukocyte recruitment, pro-inflammatory cytokine secretion and antigen capture largely by inflammatory monocytes. The induction of antigen specific Th1 cells by Carbopol was found to occur *via* a non-canonical pathway, independent of MyD88/TRIF signaling and in the absence of pattern-recognition-receptor (PRR) activation typically associated with Th1/Ig2a induction. Using multispectral fluorescence imaging (Imagestream) and electron microscopy we demonstrated that phagocytic uptake of Carbopol particles followed by entry into the phagosomal/lysosomal pathway elicited conformational changes to the polymer and reactive oxygen species (ROS) production. We therefore conclude that Carbopol may mediate its adjuvant activity *via* novel mechanisms of antigen presenting cell activation and Th1 induction, leading to enhanced IgG2a responses independent of microbial pattern recognition.

1. Introduction

Adjuvants are essential components of vaccines in which the vaccine antigen lacks robust intrinsic immunogenicity, such as those composed of recombinant or purified subunits of pathogens, or tumor antigens that may require tolerance to be broken. In recent years the adjuvant field has made breakthroughs in understanding underlying mechanisms of adjuvant activity, with the discovery of multiple pattern recognition pathways triggering innate and adaptive immune activation [1,2]. This has led to the discovery of numerous molecules with adjuvant activity that stimulate the immune system *via* defined pathways, including those triggered by toll-like receptors (TLRs) [1,2], the NLRP3 inflammasome [3,4] and IRF3 [5,6]. Some of these molecules have made their way into clinical trials and have considerable promise in vaccine development. Surprisingly, however, some of the most well-known and longstanding adjuvants, including aluminum salts 'alum', oil-in-water emulsions such as Freund's adjuvants, and MF59 appear to act by mechanisms at least partially distinct from these pathways [7–9]. Other potential modes of adjuvant action are hypothesized to include less specific activities such as the 'depot' effect by which the adjuvant sequesters antigen and releases it into the system over time, and local tissue damage resulting in release of intracellular inflammatory mediators such as ATP, nucleic acids, uric acid, IL-25 and IL-33 [6,10].

The immune-modulating activities of polyanions were first described over 30 years ago [11,12] and more recently, polyacrylic acid polymers termed carbomers have been evaluated as adjuvants in veterinary vaccines [13–18]. These reports suggest that

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carbomers are not harmful in mammals and are more effective than antigen alone. Carbopols have been combined with other adjuvant formulations such as MF59 to yield additive or potentially synergistic adaptive immune responses [19,20], and Carbopol is a component of the commercially-available adjuvant Adjuplex™ (Advanced BioAdjuvants) [21] and a licensed veterinary vaccine in pigs (Suvaxyn, Wyeth). We have previously demonstrated that Carbopol elicits strong Th1-type T and B-cell responses in mice, mediating protection from otherwise lethal influenza infection, and anti-tumor responses [22]. We observed that Carbopol did not have obvious toxicity in mice [22] or non-human primates [23], and propose that this type of polymer might have utility as a human vaccine adjuvant.

Here, we establish mechanistic insight into Carbopol's adjuvant effects, identifying strong inflammatory responses, cellular recruitment and phagocyte uptake of Carbopol, and identify phagocytosis as a key checkpoint in the immune response to Carbopol, resulting in changes to the physical properties of the adjuvant and disruption of the lysosomal pathway. We conclude that Carbopol utilizes a novel mechanism of APC activation *in vivo* resulting in potent adaptive immune responses to co-administered antigen.

2. Materials and methods

2.1. Antigens, adjuvants and immunization

HIV-1 envelope glycoprotein (Env)-based recombinant soluble gp140 (<0.05 EU/mL endotoxin) was derived from HIV-1_{97CN54} (Polymun Scientific Inc.). Pre-conjugated ovalbumin (OVA)-AF647 (Molecular Probes) was reconstituted in endotoxin-free PBS (Gibco) prior to use. A 2% (w/v) Carbopol-974p stock (Particle Sciences Inc., UK) was prepared from powder in endotoxin-free PBS, neutralized to pH 7.2 with NaOH. Carbopol preparations contained <0.05 EU/mL of endotoxin, assayed by Lonza Cologne GmbH. Alhydrogel adjuvant (Brenntag Biosector) was diluted in endotoxin-free PBS prior to injection. Balb/c, 129S6/SvEv and 129S6/SvEv.MyD88$^{-/-}$ mice were bred at the University of Oxford. C57BL/6, C57BL/6.NLRP3$^{-/-}$ and C57BL/6.Caspase1$^{-/-}$ mice were bred at Yale University. C57BL/6, C57BL/6.TRIF$^{-/-}$ and C57BL/6.MyD88$^{-/-}$TRIF$^{-/-}$ mice were bred at The Fred Hutchinson Cancer Research Center. All mice used in this study were age and sex-matched within each experiment and procedures were performed under the appropriate licenses in accordance with the UK Animals (Scientific Procedures) Act 1986 with local ethical approval.

2.2. Leukocyte phenotyping

Peritoneal leukocytes were isolated by sequential small (2 mL) and large (5 mL) volume peritoneal lavages and supernatant from small volume lavages used in cytokine/chemokine analyses. Cell fractions from both lavages were pooled and analyzed by flow cytometry. The absolute numbers of B-cells (CD11b$^{-/int}$CD19^{+}), T-cells (CD11b^{-}CD3^{+}), monocytes (CD11b^{hi}Ly6C^{++}Ly6G^{-}F4/80^{int}), macrophages (CD11b^{+}F4/80^{hi} Ly6G^{-}Ly6C^{-}), neutrophils (CD11b^{+}Ly6G^{hi}Ly6C^{+}F4/80^{-}), eosinophils (CD11b^{+}Ly6C^{lo}Ly6G^{int}F4/80^{lo}SSC^{hi}), and dendritic cells (DC) (CD11b$^{-/int}$CD11c^{+}F4/80$^{-/lo}$MHC-II^{hi}) were determined. To reduce non-specific antibody binding, cells were pre-incubated in Mouse Fc-Block (BD Biosciences). Flow cytometry was performed using either FACSCalibur (BD Biosciences) or CyAN ADP cytometers (Beckmann Coulter, USA) and data analyzed *via* FloJo software (TreeStar Inc., USA).

2.3. Antibody/cytokine/chemokine detection

Serological analyses for antigen-specific antibodies were performed as previously described [22]. Supernatants were separated from either peritoneal lavage or cultured cells and cytokine concentrations determined *via* Bio-plex array (Bio-Rad Laboratories) or ELISA (eBioscience). Analyses were performed following manufacturer's instructions and cytokine concentrations calculated using standard curves generated within the same assay. Antigen-specific T-cell IFNγ secretion/accumulation was assessed in splenocytes cultured in the presence of gp140 (16 μg/mL) over 3–5 days. Secreted IFNγ was detected by ELISA (eBioscience) and the frequencies of antigen specific IFNγ producing T-cells were detected by IFNγ cytokine secretion assay (Miltenyi Biotec).

2.4. In vitro *stimulation*

Murine RAW-Blue and human THP-1-CD14-Blue (Invivogen) reporter cells expressing NFκB/AP-1 transcription factor-induced embryonic alkaline phosphatase (SEAP) were used to detect PRR stimulation. Cells were cultured for 24 h in the presence of PBS, Carbopol or a variety of PRR ligands, supernatants harvested and co-incubated with SEAP detection reagent Quantiblue (Invivogen). Inflammasome activation was assessed after overnight incubation in the presence or absence of 25 ng/mL *Escherichia coli* LPS (Sigma–Aldrich, USA), followed by extensive washing to remove trace LPS prior to *in vitro* adjuvant stimulation (4 h, or (ATP) 30 min). Supernatants were frozen and cells lysed in RIPA buffer (Life Technologies) containing protease inhibitors. Lysates were directly loaded in Laemmli buffer and gels semi-dry transferred to nitrocellulose membranes (Amersham Biosciences, UK). Membranes were blocked with 5% milk in PBS (Oxoid, UK) and caspase-1 detected by polyclonal rabbit anti-caspase-1 antibody (Santa Cruz, USA) and anti-rabbit IgG-HRP (Serotec, USA).

Please refer to the supplementary materials for additional methods.

3. Results

3.1. Carbopol triggers immune cell recruitment and potently induces pro-inflammatory chemokines and cytokines

To investigate the potential mode of action of Carbopol, we compared early innate immune responses induced by Carbopol with those induced by Alhydrogel, which elicits a strong Th2/IgG1-associated immune bias [24]. Since cellular recruitment is increasingly recognized as having a major influence on adjuvanticity and adaptive immune outcome in response to adjuvants [4,6,8,10,25–27], we assessed Carbopol-induced chemokine and cytokine secretion using the intraperitoneal (i.p.) administration model. Although this does not represent a human immunization route, it nevertheless represents a useful model for mechanistic analysis [8,21]. Strong responses were detected in Carbopol recipients (Fig. 1), with significant induction of IL-1β, IL-6, G-CSF, KC, MIP-2, MCP-1 and RANTES (Fig. 1A–G). These chemokine trends were shared by Alhydrogel-adjuvanted mice, which also displayed significant but less potent induction of IL-6, G-CSF, KC, and MCP-1. One notable exception was the eosinophil recruitment factor Eotaxin, secreted at high levels by Alhydrogel-treated mice, but absent in Carbopol recipients (Fig. 1H). By contrast, the pattern of cytokine secretion associated with T-cell polarization was strikingly different between Carbopol and Alhydrogel groups (Fig. 1I–K). Alhydrogel recipients expressed significantly elevated levels of the Th2-associated cytokines IL-4 and IL-5, whereas Carbopol conversely elaborated significant quantities

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