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Formulation of aluminum hydroxide adjuvant with TLR agonists poly(I:C) and CpG enhances the magnitude and avidity of the humoral immune response

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ABSTRACT

Subunit vaccines generally require adjuvants to achieve optimal immune responses. Toll-like receptor (TLR) agonists are promising immune potentiators, but rapid diffusion from the injection site reduces their local effective concentration and may cause systemic reactions. In this study, we investigated the potential of aluminum hydroxide adjuvant (AH) to adsorb the TLR3 agonist poly(I:C) and TLR9 agonist CpG and compared the effect of the combination adjuvant on the immune response with either the TLR agonists or AH alone in mice. Poly(I:C) and CpG readily adsorbed onto AH and this combination adjuvant induced a stronger IgG1 and IgG2a immune response with a significant increase of antibody avidity. The combination adjuvant enhanced antigen uptake and activation of dendritic cells *in vitro*. It induced an inflammatory response at the injection site similar to AH but without eosinophils which are typically observed with AH. A distinctive antigen-containing monocyte/macrophage population with an intermediate level of CD11c expression was identified in the draining lymph nodes after immunization with TLR agonists and the combination adjuvant. Injection of the combination adjuvant did not induce an increase of TNFα and CXCL10 in serum in contrast to the injection of soluble TLR agonists. These results indicate that this combination adjuvant is a promising formulation to solve some of the unmet needs of current vaccines.

1. Introduction

Adjuvants are important components of modern vaccines because purified and molecularly defined antigens are increasingly preferred in formulations to enhance their efficacy, consistency and safety, but lack immunogenicity [1,2]. Immunostimulatory microbial molecules are attractive candidates for the development of new adjuvants. Microbial pathogens activate the immune response through the recognition of conserved microbial molecules by innate immune cells via germ-line encoded receptors, including Toll-like receptors (TLRs), NOD-like receptors (NLR) and retinoic acid inducible gene-I-like receptors (RLRs). TLRs are expressed on the cell membrane and in endosomal compartments, whereas NLRs and RLRs are present in the cytoplasm of cells. Ligands for these receptors are potent activators of immune cells and promising new adjuvant candidates [3,4]. The activation of these receptors induces the expression of cytokines and costimulatory molecules that activate and direct the adaptive immune response. The binding of TLR ligands to TLRs results in signal transduction that involves the adaptor molecules MyD88 and TRIF. Most TLRs use MyD88, whereas TLR4 engages both MyD88 and TRIF, and TLR3 exclusively signals in a MyD88-independent manner through TRIF [5]. Signaling through MyD88 and TRIF activates different downstream signaling pathways resulting in different patterns of gene activation. This results in a synergistic effect when mouse and human macrophages and dendritic cells are cultured with combinations of TLR ligands that activate cells in a MyD88-dependent and MyD88-independent manner [6–8].

The ligands for TLRs are typically small molecules that diffuse away rapidly from the injection site decreasing the local dose available for activation of innate immune cells and potentially resulting in systemic activation of TLRs and toxicity. Encapsulation, emulsification, or adsorption to particles can allow for local delivery of TLR ligands to antigen-presenting cells while limiting their biodistribution and avoiding systemic reactions [9–12]. Incorporation of antigen in a particulate formulation will deliver antigen to

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the same antigen-presenting cells that are activated by the TLR ligands which is required for enhanced antigen processing and presentation [13]. Aluminum adjuvants can be used as a platform to deliver both TLR ligands and antigen to antigen presenting cells. They have been used for nearly a century in human vaccines and have demonstrated excellent safety and tolerability [14,15]. Aluminum adjuvants have a very large adsorptive surface which potentially allows adsorption of multiple antigens and TLR ligands [16]. In addition, aluminum adjuvants activate the NLRP3 inflammasome in dendritic cells and macrophages which leads to the secretion of IL-1β and IL-18, providing a complementary stimulatory pathway to TLR ligands [17]. The combination adjuvant AS04, which contains an aluminum adjuvant and the TLR4 agonist monophosphoryl lipid A, is used in licensed vaccines against hepatitis B and human papilloma virus [18]. Aluminum adjuvants with other single TLR4, TLR7 and TLR9 agonists are in phase 1 and 2 clinical trials [16]. We investigated the formulation and immunostimulatory effect of a combination adjuvant comprised of aluminum hydroxide adjuvant (AH) and two TLR ligands, poly(I:C) and CpG. Poly(I:C) is structurally similar to double stranded RNA that binds TLR3 resulting in the recruitment of TRIF and activation of the NF-kB and IRF3/7 signaling pathways [19,20]. CpG are oligodeoxynucleotides with unmethylated CpG motifs that bind TLR9 resulting in activation of the NF-kB signaling pathway in a MyD88-dependent manner [21]. Both nucleotide ligands have a negative charge that enables electrostatic adsorption to AH which carries a positive surface charge at neutral pH [14]. The complex of AH with poly(I:C) and CpG activates complementary pathways in dendritic cells and is expected to demonstrate robust and synergistic enhancement of the immune response. Aluminum adjuvants facilitate the phagocytosis of adsorbed molecules and this will target the nucleotide ligands to their endosomal receptors. Furthermore, we expected co-adsorption of antigen with TLR agonists to AH to enhance the uptake of antigen by dendritic cells. Here, we report on the formulation of this triple combination adjuvant and its effect on dendritic cells *in vitro*, and the effect on the magnitude and quality of the antibody response to co-adsorbed antigen.

2. Materials and methods

2.1. Mice and materials

Five to seven week old female BALB/c mice from Envigo (Indianapolis, IN) were housed in the animal housing facility of Purdue University with free access to food and water. Mice were acclimated for a week before any procedures. All procedures were approved by the Institutional Animal Care and Use Committee of Purdue University. Aluminum hydroxide adjuvant (Rehydragel HPA) was obtained from Chemtrade (Berkeley Heights, NJ). Low molecular weight poly(I:C) and CpG ODN 1826 were purchased from Invivogen (San Diego, CA). Proteins included ovalbumin (OVA; Invivogen), human serum albumin (HSA; Sigma), and recombinant protective antigen (rPA; List Biologicals, Campbell, CA).

2.2. Adsorption of TLR ligands and antigen to AH

AH was diluted to 1 mg Al^{3+}/mL in Tris buffered saline (pH 7.4) and mixed with different concentrations of poly(I:C) or/and CpG for 1 h. After centrifugation at 10,000 rpm for 5 min, supernatants were collected. The concentration of poly(I:C) in the supernatant was measured with a Quant-iT^{TM} RNA assay kit (Thermo Fisher Scientific) and CpG with a NanoDrop One microvolume spectrophotometer. To test adsorption of antigen by the combination adjuvant, OVA was mixed with the combination adjuvant for an hour. The concentration of OVA in the supernatant was measured with a Micro BCA^{TM} protein assay kit (Thermo Fisher Scientific).

2.3. Transmission electron microscopy

Droplets of AH (500 μg/mL in Tris-buffered saline) or combination adjuvant (500 μg/mL AH with 125 μg/mL poly(I:C) and 25 μg/mL CpG in Tris-buffered saline) were dried on a 400 mesh carbon-coated grid. The grids were imaged under a FEI Tecnai G2 20 transmission electron microscope.

2.4. Immunization

Mice were injected in each calf muscle with 50 μL OVA (Invivogen, San Diego, CA), rPA, or Alexa Fluor-647-labeled OVA (Thermo Fisher Scientific) in Tris-buffered saline (pH 7.4), with or without adjuvants. The concentrations of antigens and adjuvants used were 50 μg/mL OVA, 20 μg/mL rPA, 1.0 mg Al^{3+}/mL AH, 250 μg/mL poly (I:C), and 50 μg/mL CpG. Muscle and draining iliac lymph nodes were collected from mice at the indicated times after immunization. Cells in the iliac lymph node were released through treatment with 0.02% (w/v) collagenase D (Roche Diagnostics) before labeling for flow cytometry. For detection of antibodies, mice were injected with the same formulation three weeks later, and euthanized after two weeks.

2.5. ELISA

Serum cytokines were measured with a CXCL10 ELISA kit (R&D Systems, Minneapolis, MN) and a TNF-α ELISA kit (eBioscience, San Diego, CA). Serum antibody titers were determined as described previously [22]. For antibody avidity measurement, two sets of duplicates were prepared for each diluted serum sample. After incubation with serum, one set was treated with 7 M urea (Sigma) while the other set was treated with PBST for 10 min before secondary antibody was added. The avidity index was calculated as OD_{450} (urea-treated sample)/OD_{450} (PBST- treated sample).

2.6. Antigen uptake by bone marrow-derived dendritic cells (BMDCs)

BMDCs [23] were seeded in six well pates (Corning Cellgro, Corning, NY) at a concentration of 10^6 cells/mL with a volume of 2 mL per well in complete RPMI medium supplemented with 5% FBS. FITC-labeled antigens [23] were added at a final concentration of 2 μg/mL with or without 50 μg/mL AH, 10 μg/mL poly(I:C), and 2 μg/mL CpG. After two hours, the cells were washed with ice cold PBS to remove extracellular antigens. The cells were released from the plate by treatment with 0.25% trypsin/2.21 mM EDTA-containing HBSS (Corning Cellgro), and fixed with 4% paraformaldehyde followed by immunofluorescence staining on a slide or resuspension in PBS for flow cytometry staining.

2.7. Activation of BMDCs

BMDCs were incubated with adjuvants were added to a final concentration of 50 μg/mL AH, 10 μg/mL poly(I:C), or 2 μg/mL CpG. After 48 h, supernatants were harvested and the concentrations of cytokines were measured by ELISA (eBioscience, San Diego, CA). The BMDCs were resuspended in PBS containing 2 mM EDTA and stained with Zombie Violet dye (Biolegend). The cells were washed, blocked with 1% normal rabbit serum, and labeled with antibodies including Alexa Fluor (AF) 488-anti-mouse CD11c, PE-anti-mouse I-A/I-E, and AF488-anti-mouse CCR7 (BioLegend), PE-anti-mouse CD80, or PE-anti-mouse CD86 (eBioscience). The labeled cells were resuspended in 2% paraformaldehyde and ana-

lyzed in a BD Canto II flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed by FlowJo software (Flowjo, Eugene, OR).

2.8. Immunofluorescence of the injection sites

Injection sites collected at 6 h, 1 or 7 days post immunization were fixed in 4% paraformaldehyde for 3 h and dehydrated in 30% sucrose solution overnight at room temperature as described [22]. Tissue samples were embedded in Optimal Cutting Temperature medium, snap frozen in liquid nitrogen, and sectioned at 7 μm thickness. Cryosections were blocked with 1% BSA and labeled with phenol red-containing HBSS (for eosinophils), biotin-anti-Mac-2, biotin-anti-F4/80, biotin-anti-I-A/I-E, biotin-anti-Ly6G (all from Biolegend), or biotin-anti-CD11b (Invitrogen, Carlsbad, CA) followed by AF488 or AF549-conjugated streptavidin (Jackson Immunoresearch). Slides were coverslipped with Prolong Gold antifading with DAPI (Invitrogen). Cells were counted under a Nikon E400 fluorescent microscope and images were obtained with a Nikon A1R multiphoton confocal microscope.

2.9. Statistical analysis

The statistical significance of differences between treatments was determined using one way ANOVA with Tukey's post hoc analysis. Differences between serum antibody titers were analyzed after log10 transformation.

3. Results

3.1. Adsorption of poly(I:C), CpG, and antigen to AH

Increasing concentrations of the TLR ligands were mixed with AH for one hour to determine the degree of adsorption. Poly(I:C) and CpG adsorbed strongly to AH with an adsorptive capacity of approximately 1500 μg/mg Al^{3+} for poly(I:C) and 500 μg/mg Al^{3+} for CpG (Fig. 1). Experiments were conducted with a combination adjuvant formulated with 250 μg poly(I:C) and 50 μg CpG per mg Al^{3+}. At these concentrations both poly(I:C) and CpG were completely adsorbed. The protein antigen OVA was added to the combination adjuvant at a dose of 50 μg/mg Al^{3+}, and was completely adsorbed at this dose. The morphology of AH and the combination adjuvant was evaluated by transmission electron microscopy (Fig. 2). The combination adjuvant maintained the needle-like crystal structure of AH. Electron dense particles at a size of ~30 nm were associated with AH and we speculate that these represent aggregates of the nucleotides.

3.2. Combination adjuvant induced a stronger IgG1 and IgG2a response, and enhanced avidity of antigen-specific antibodies

Mice were injected with rPA alone or with AH, TLR agonists, or the combination adjuvant. All adjuvants significantly enhanced the titer of rPA-specific IgG1 (Fig. 3A), but the combination adjuvant

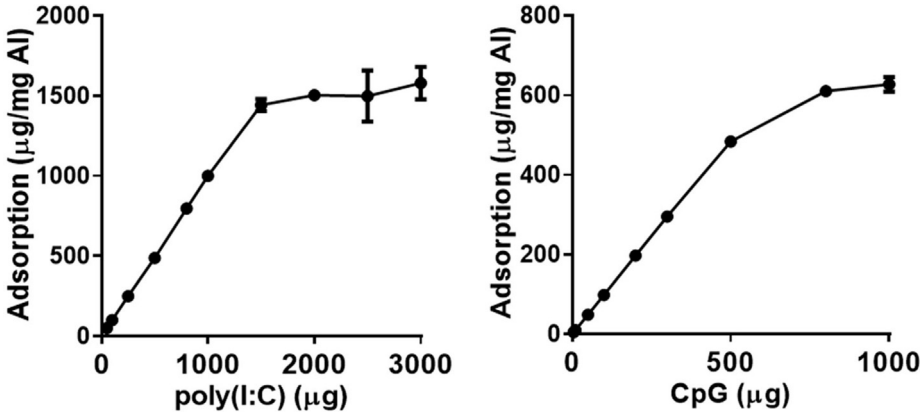

Fig. 1. Adsorption of poly(I:C) and CpG to AH. Different concentrations of poly(I:C) (A) and CpG (B) were mixed with 1 mg/mL AH and the amount of polynucleotides adsorbed onto AH was calculated. Data represent the mean ± SEM of four samples.

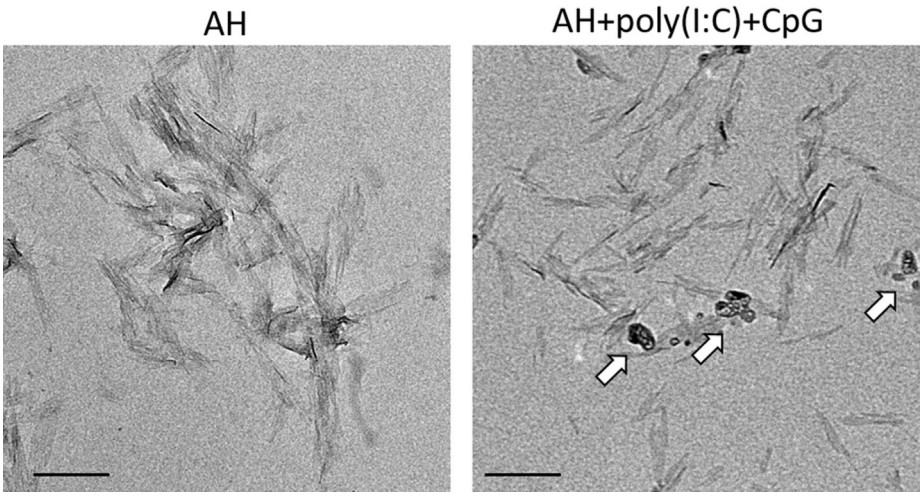

Fig. 2. Transmission electron microscopy of AH and the combination adjuvant. Small particles were found to be associated with the combination adjuvant (white arrows). Bar = 200 μm.

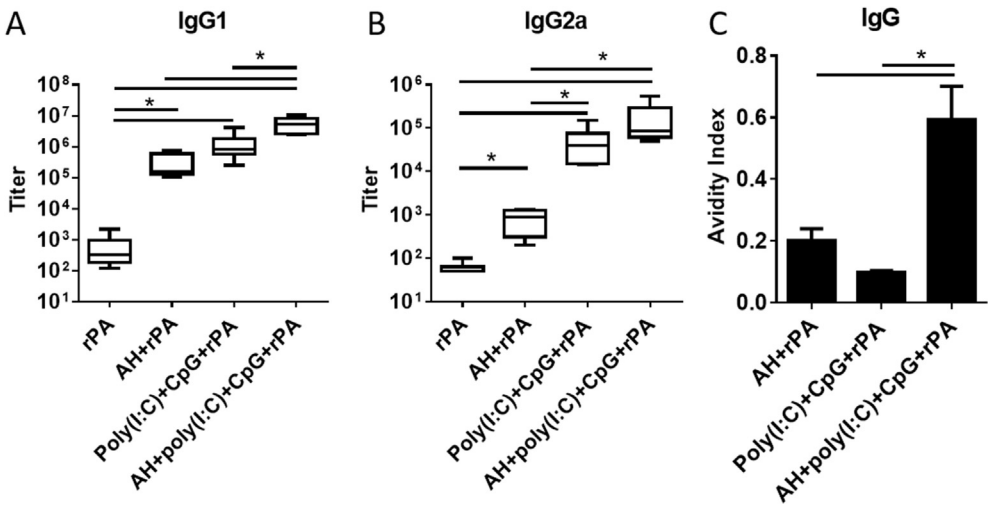

Fig. 3. Effect of the combination adjuvant on the antibody response. Anthrax rPA antigen was injected either alone or with the indicated adjuvants, and the titers of rPA-specific IgG1 (A) and IgG2a (B) in the serum were measured by ELISA. Boxes indicate 5–95% confidence interval and median. (C) Avidity of rPA-specific IgG. Data represent mean ± SEM of six samples. * $p < 0.05$.

induced a higher titer compared with either AH or TLR agonists alone. Recombinant PA alone and rPA combined with AH did not induce a significant rPA-specific IgG2a titer (Fig. 3B). In contrast, the TLR agonists and combination adjuvant significantly enhanced the rPA-specific IgG2a titer. In addition to the quantity of rPA-specific antibodies, we determined the avidity. The avidity of rPA-specific IgG of the combination adjuvant group was significantly higher than that of the AH and TLR agonists groups (Fig. 3C). Similar results were observed with OVA as antigen (Fig. S1).

3.3. Combination adjuvant enhanced antigen uptake by BMDCs

When BMDCs were incubated with FITC-OVA alone, there was significant uptake of OVA (Fig. 4A). Formulating OVA with AH did not affect antigen uptake, whereas incubation with TLR agonists decreased the number of OVA-containing cells. However, the percentage of OVA-positive cells increased when OVA was adsorbed to the combination adjuvant (Fig. 4A). The mean fluorescent intensity (MFI) indicated that the combination adjuvant induced greater per cell uptake of OVA compared with the other adjuvants (Fig. 4B). We also tested the effect of different adjuvants on the uptake of human serum albumin (HSA), an antigen that is barely taken up in soluble form (Fig. S2). While TLR agonists did not affect the uptake of HSA, an increased percentage of DCs internalized HSA when formulated with AH, and this increased further with the combination adjuvant (Fig. S2). The increased uptake of antigen with the combination adjuvant by DCs was confirmed by confocal microscopy (Fig. S2).

3.4. Activation of dendritic cells by the combination adjuvant

The effect of different adjuvant formulations on the activation of DCs was determined by the expression of costimulatory molecules and secretion of cytokines. AH had minimal effect on the expression of CD80 and CD86, whereas TLR agonists alone and in combination with AH significantly enhanced their expression (Fig. 5). The TLR agonists increased the expression of CD86 more strongly alone than in combination with AH. The cytokines IL-1β, IL-6, IL-12, IL-23, and TNF-α are pro-inflammatory and play key roles in the differentiation of CD4 T cells. AH did not induce any of these cytokines, whereas the TLR agonists and combination adjuvant induced secretion of IL-23, IL-6, TNF-α, and IL-12 (Fig. 6). The TLR agonists induced more IL-6, IL-12, and IL-23 secretion than the combination adjuvant. Neither AH nor TLR agonists induced secretion of IL-1β, and only the combination adjuvant activated DCs to release IL-1β into the supernatant (Fig. 6).

3.5. AH and combination adjuvant induced inflammation at the injection site

Induction of an inflammatory response enhances the immune response since it recruits antigen presenting cells and provides signals for activation of the adaptive immune response [1,2]. The degree and quality of the inflammatory response affect the magnitude and type of immune response. Very few inflammatory cells were present at the injection site after injection of TLR agonists only (Fig. 7 and Fig. S3), presumably because TLR agonist are small molecules that diffuse quickly from the injection site. In contrast, injection of AH and the combination adjuvant caused significant inflammation with accumulation of inflammatory cells including neutrophils, antigen presenting cells (MHCII+), and macrophages. A notable difference between AH and the combination adjuvant was that the AH injection site contained eosinophils which were absent after injection of the combination adjuvant (Fig. 7 and Fig. S3). Recruitment of other inflammatory cells was similar between AH and the combination adjuvant. There were more Mac-2^+ cells than F4/80^+ cells, indicating heterogeneity in the macrophage population that accumulated at the injection site. Mac-2 is a marker of activated macrophages, whereas the expression of F4/80 decreases upon activation [24,25].

3.6. TLR agonists and combination adjuvant induced the appearance of a distinct population of antigen-containing cells in the draining lymph node

After taking up antigen at the injection site, antigen presenting cells will migrate to the draining lymph node to present processed antigenic peptides to T cells and initiate the adaptive immune response. We investigated the appearance of antigen-containing cells in the draining iliac lymph node following injection of FITC-OVA mixed with different adjuvants. There was no difference in the percentage of antigen-containing cells among the myeloid cells ($SSC^{medium\text{-}high}FSC^{medium\text{-}high}$) in the lymph node between the three

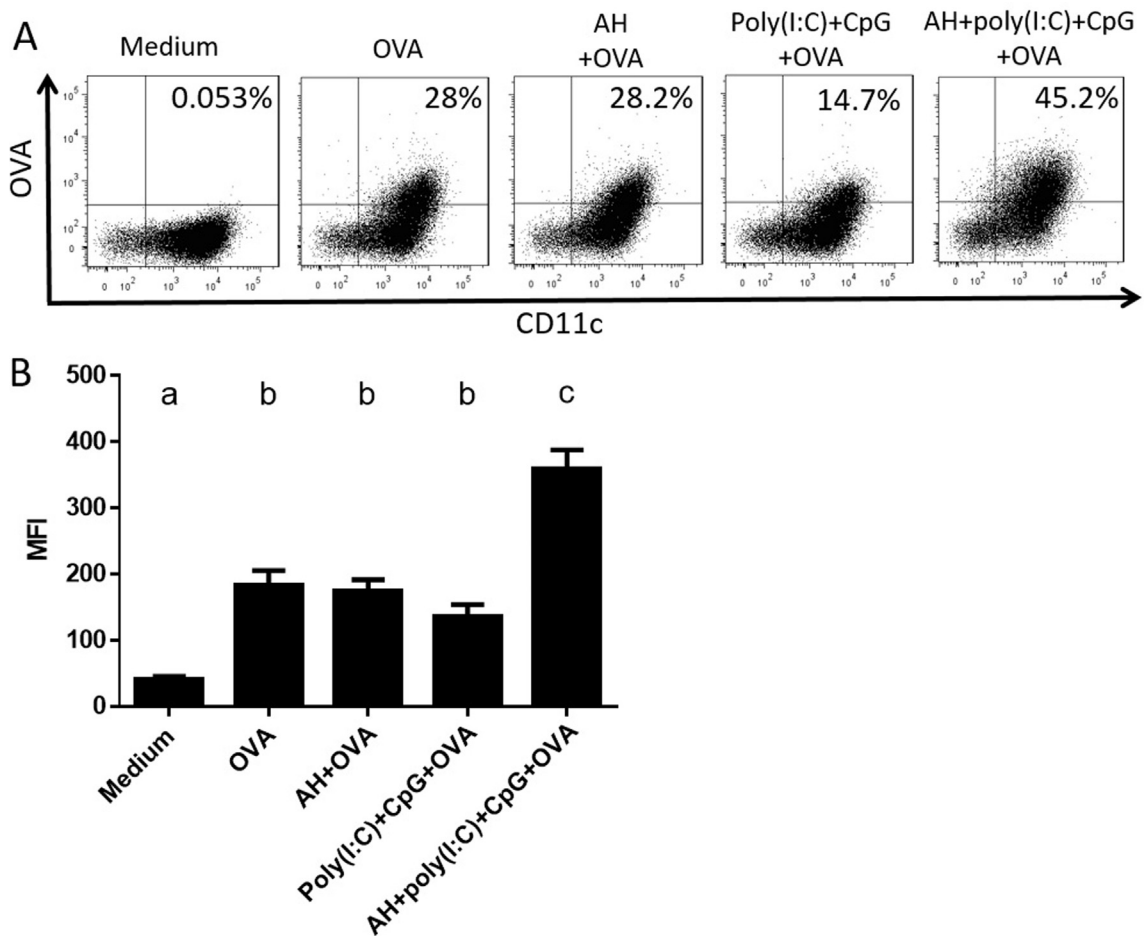

Fig. 4. Effect of the combination adjuvant on the uptake of OVA by dendritic cells. Dendritic cells were incubated with FITC-labeled OVA alone or mixed with different adjuvant formulations and the uptake was assessed by flow cytometry. (A) Representative dot plots; (B) mean fluorescence intensity (MFI). Bars indicate the mean + SEM of four independent experiments. Different letters indicate a significant difference at $p < 0.05$.

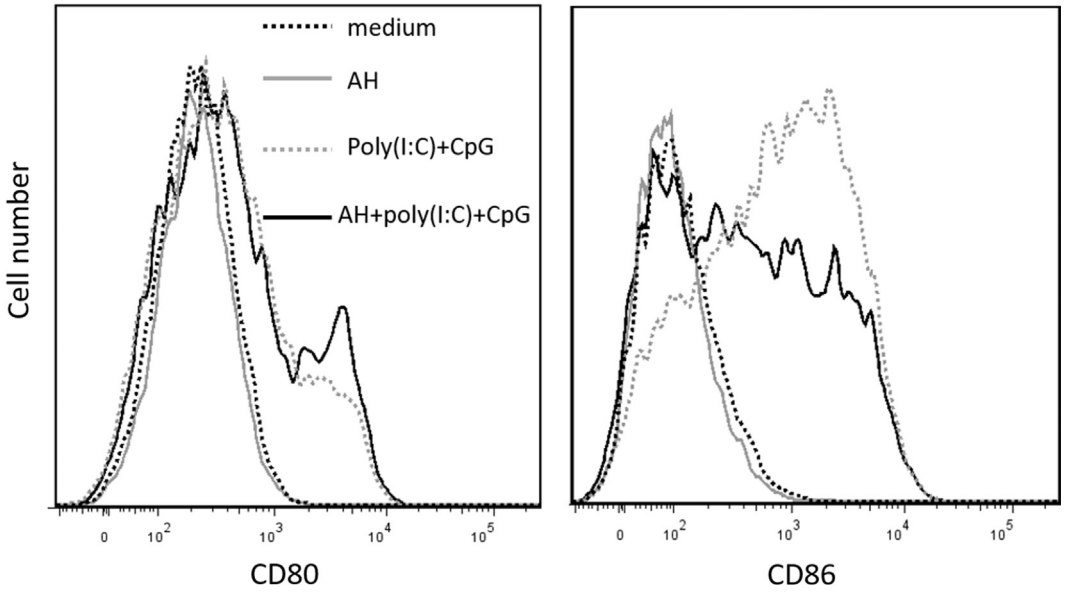

Fig. 5. The combination adjuvant and TLR agonists increased expression of costimulatory molecules by dendritic cells. Dendritic cells were incubated with medium only or with different formulations of adjuvants and the expression of CD80 (A) and CD86 (B) was examined by flow cytometry. Data are representative of three independent experiments.

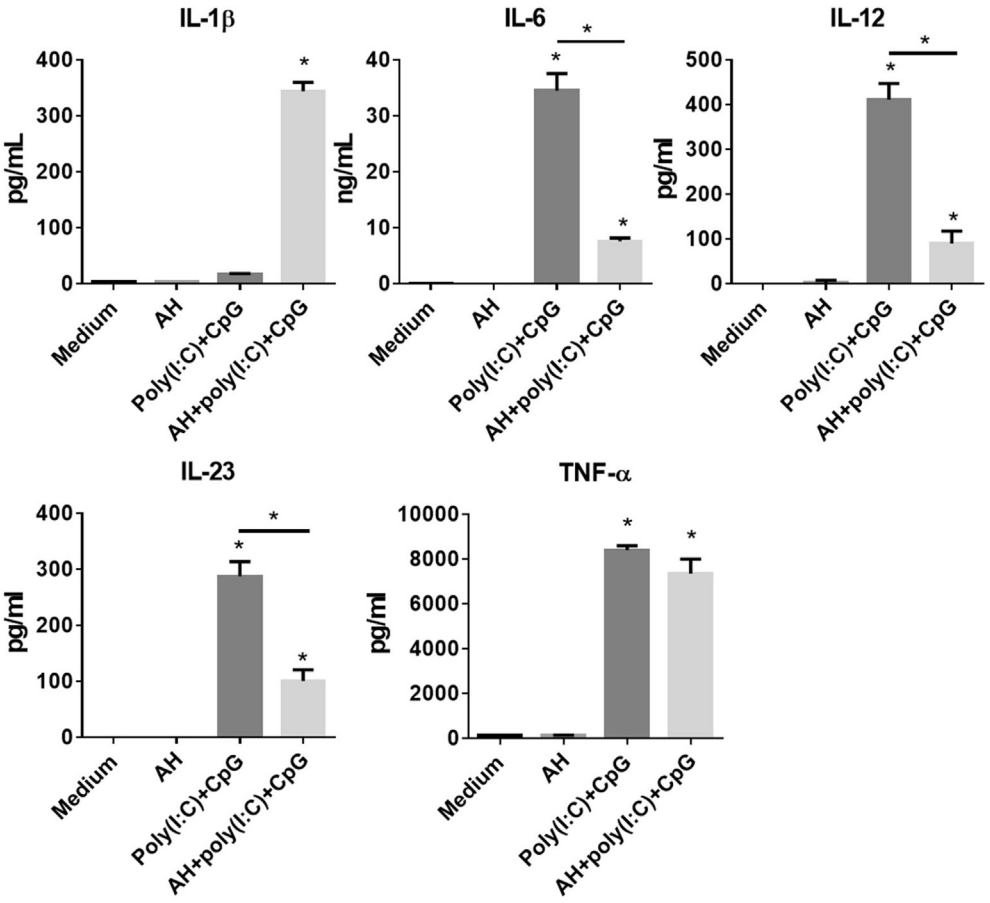

Fig. 6. Cytokine secretion by dendritic cells. Dendritic cells were incubated with medium only or with different adjuvants, and cytokines were quantified by ELISA. Data represent the mean ± SEM of four samples. *: $p < 0.05$.

adjuvant groups (Fig. 8A). The TLR agonists and combination adjuvant induced some enlargement of the lymph nodes compared with the other two formulations, but there was no significant difference in the total number of antigen-containing cells in the lymph node (data not shown).

Based on the expression of MHCII and CD11c, antigen-containing cells in the lymph node were separated into three subpopulations (Fig. 8B): $MHCII^{high}CD11c^{medium}$ migratory DC (MigDC); $MHCII^{medium}CD11c^{high}$ lymphoid tissue resident DC (LTDC); and $MHCII^{medium\text{-}low}CD11C^{medium\text{-}low}$ monocytes and macrophages [26,27]. Although there was no difference in the total number of antigen-containing myeloid cells in the lymph node among the different vaccine formulations, the monocyte/-macrophage population made up a larger fraction of OVA-containing cells following injection of OVA with TLR agonists and the combination adjuvant compared with OVA alone or OVA with AH. This population of cells also expressed a higher level of CD11c compared with the monocytes and macrophages identified in AH immunized mice (Fig. 8C).

3.7. The combination adjuvant does not increase systemic production of cytokines

Although TLR agonists induce a strong and balanced immune response, widespread diffusion of small molecule agonists after injection can cause systemic release of proinflammatory cytokines and toxicity which prevents their direct application as vaccine adjuvants [10,11,28]. To study the effect of different adjuvants on systemic inflammation, mice were immunized with OVA either alone or formulated with different adjuvants. Six hours after immunization, CXCL10 and TNF-α were detected when OVA was formulated with TLR agonists but not with any other formulations, including the combination adjuvant (Fig. 9).

4. Discussion

In this study, we demonstrate the utility of AH, a safe and widely used adjuvant, as the basis for a combination adjuvant that incorporates two different TLR agonists, poly(I:C) and CpG. The immune response induced with the combination adjuvant was stronger than that obtained with TLR agonists or AH alone. The formulation takes advantage of the positive surface charge of AH at neutral pH and its large adsorptive capacity which allow electrostatic adsorption of multiple negatively charged molecules. This permits the delivery of the polynucleotide TLR agonists and antigen to the same antigen presenting cells, a requirement for enhanced antigen processing and presentation [13].

The immune response induced by AH is biased towards humoral immunity and provides protection through the induction of neutralizing antibodies. Although licensed aluminum adjuvanted vaccines are effective for protection of the general population, a stronger adjuvant may be necessary for certain subgroups with a less responsive immune system such as the elderly. A stronger adjuvant may also be required for therapeutic vaccines for treatment of cancer and chronic infectious diseases that require an effective cell-mediated immune response and complement-fixing antibodies. Here we demonstrate that the formulation of AH with TLR ligands poly(I:C) and CpG induced a stronger and

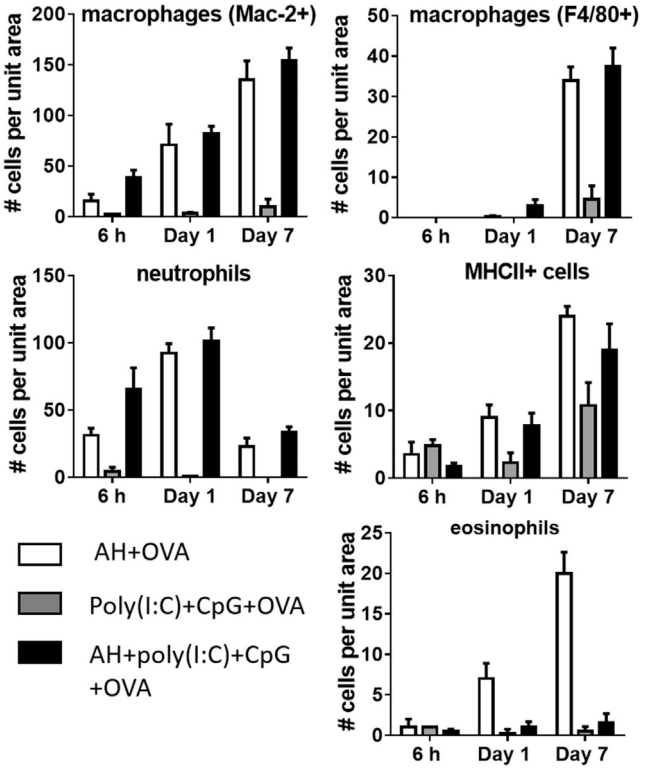

Fig. 7. Accumulation of inflammatory cells the injection site after injection of different adjuvant formulations. Injection sites were harvested at indicated points after immunization and labeled with antibodies and chemicals to identify inflammatory cells. Data represent the mean + SEM of four samples.

qualitatively different antibody response compared with AH alone based on a 10-fold increase of IgG1 and a more than 100-fold increase of IgG2a as well as a marked increase of antibody avidity. In mice, IgG1 is associated with a Th2 response and IgG2a with a Th1 response [29,30]. Functionally, IgG2a is more effective than IgG1 at activating the complement system [31–33] and binds to the high affinity FcγRI and FcγRIV expressed on neutrophils, macrophages and dendritic cells which do not bind IgG1 [34].

The combination adjuvant in this study consisted of three components that activate different cellular pathways in DCs. Although the exact mechanisms by which aluminum adjuvants enhance the immune response remains unclear, *in vitro* studies have clearly demonstrated that these adjuvants activate DCs to release IL-1β, a potent inflammatory mediator [35,36]. The secretion of IL-1β is a two-step process initiated by activation of the NF-κB signaling pathway by molecules such as TLR agonists which results in the transcription and translation of pro-IL-1β [37]. Processing of pro-IL-1β by the proteolytic enzyme caspase-1 is necessary for the release of biologically active IL-1β from the cell [37]. Caspase-1 is a component of the NLRP3 inflammasome which is activated by aluminum adjuvant in DCs and macrophages [38–42]. The lack of IL-1β in DC cultures incubated with either TLR agonists or AH, and the presence of IL-1β after incubation with the combination adjuvant is consistent with the requirement for two signals. Incubation of DCs with AH had no effect on the expression of costimulatory molecules or the secretion of other cytokines as previously reported [35,43]. The combination adjuvant induced less IL-6, IL-12 and IL-23 compared with the TLR agonists by themselves. This may be explained in part by the adsorption of the TLR agonists to the AH particles reducing the effective concentration of the agonists in the static cell culture system. Following injection of the vaccine in vivo, the soluble TLR agonists would diffuse quickly from the injection site and adsorption to AH would actually increase the effective concentration and target both agonists to DCs. In addition, adsorption of the polynucleotides to solid particles may affect the interaction with their receptors as demonstrated for CpG [44]. The surface density of CpG adsorbed onto nano- or microparticles altered the quality and magnitude of signaling and the biological effects. Furthermore, AH has a direct negative effect on IL-12 secretion by DCs [45]. This effect was largely reversed by inhibition of phosphatidylinositol-3-kinase, consistent with activation of this pathway by AH [46].

The adsorption of soluble protein antigens to aluminum adjuvants increased the uptake of antigens by dendritic cells as previously reported [47,48]. In contrast, the TLR agonists had no effect on the uptake of HSA, and actually decreased the uptake of soluble OVA. Signaling via TLRs can initially increase antigen uptake, but this decreases as DCs undergo maturation [49]. Soluble OVA is taken up by DCs via the mannose receptor and to lesser extent via pinocytosis and the scavenger receptor [50,51]. The smaller uptake upon incubation with poly(I:C) and CpG is consistent with the report that maturation of DCs following stimulation with TNF and LPS reduced the expression of the mannose receptor and inhibited macropinocytosis [52]. In addition, poly(I:C) can bind to scavenger receptors and may reduce uptake of OVA independent of DC activation [53]. The combination adjuvant induced the greatest uptake of protein antigens suggesting that the particulate form facilitates rapid uptake of antigen through phagocytosis before TLR-induced DC maturation shuts down the uptake process.

Particulate adjuvants such as aluminum adjuvants and emulsions induce the expression of chemokines and cytokines at the site of injection [22,54,55]. This allows the recruitment and activation of inflammatory cells including antigen presenting cells and generates signals necessary for the activation and maturation of antigen presenting cells and induction of an adaptive immune response in the draining lymph node. The magnitude and type of inflammatory signals that antigen presenting cells receive at the injection site affects the following immune response. Such inflammatory reaction should not be excessive and not become systemic causing clinical signs such as fever and general malaise. Injection of aluminum adjuvant alone or with poly(I:C) and CpG induced a moderate local inflammatory reaction which differed qualitatively in the nearly complete lack of eosinophils in mice injected with the combination adjuvant. As eosinophils are a hallmark of type 2 inflammation, the lack of these cells indicates downregulation of type 2 inflammatory reactions by the TLR agonists. Poly(I:C) and CpG alone did not induce a local reaction, but increased TNF and CXCXL10 in serum indicative of a systemic inflammatory reaction. A systemic reaction was not observed when poly(I:C) and CpG were adsorbed to AH. This demonstrates that adsorption of the polynucleotides focused their proinflammatory activity locally, allowing recruitment and activation of antigen presenting cells to the site of injection, and prevented an unwarranted systemic response.

The combination adjuvant induced a marked increase of the avidity of the OVA and HSA-specific antibodies compared with AH alone or the soluble TLR agonists. Germinal centers are essential for affinity maturation following vaccination or infection [56]. The formation of germinal centers is enhanced by TLR3 and TLR9 ligands and involves signaling of TLRs in B cells and dendritic cells [57,58]. Following injection of OVA with the combination adjuvant, most antigen-positive cells were present in the monocyte/macrophage fraction of myeloid cells in the draining lymph node. These cells likely represent monocyte-derived dendritic cells which promote the differentiation of T follicular helper cells in an IL-6-dependent manner [59]. These data suggest that the combination adjuvant induced high avidity antibodies through the recruitment and maturation of monocyte-derived antigen-presenting

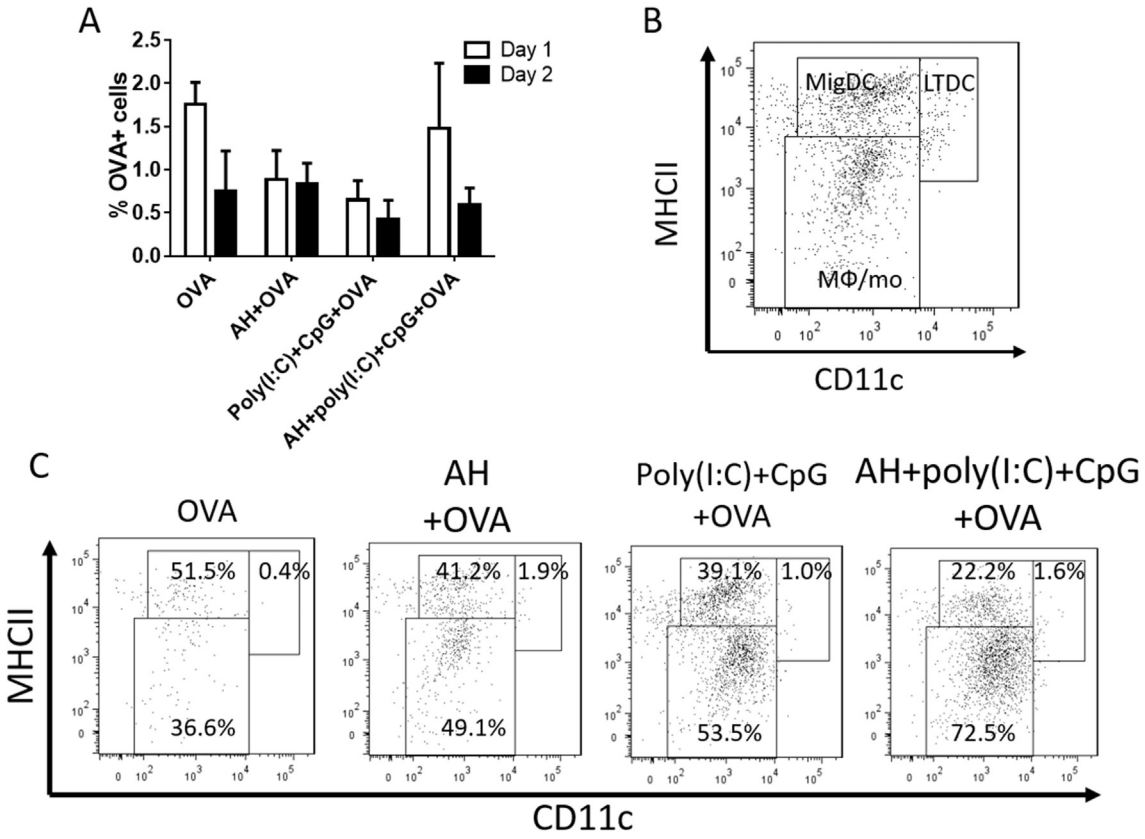

Fig. 8. Effect of the combination adjuvant on antigen transport to the draining lymph node. Iliac lymph nodes were collected after calf muscle injection of different vaccine formulations. (A) Percentages of myeloid cells in the iliac lymph nodes that contained OVA. Bars represent the mean ± SEM of five independent experiments. (B) Three subtypes of OVA-containing antigen presenting cells were identified in the iliac lymph node: MigDC-migratory dendritic cells, LTDC-lymphoid tissue dendritic cells, MΦ/Mo-macrophages and monocytes. (C) OVA-containing antigen presenting cells in the iliac lymph node two days after immunization. Data are representative of five independent experiments.

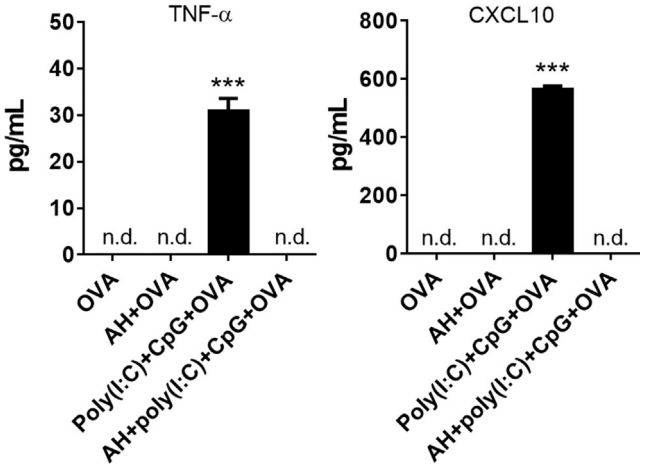

Fig. 9. Combination adjuvant does not induce an increase of the serum concentration of TNF-α and CXCL10. Serum samples were collected six hours after injection of different adjuvants. Data represent the mean ± SEM of four samples. n.d. – not detectable.

cells leading to enhanced function of germinal center T follicular helper cells.

In conclusion, we have shown here that TLR agonists poly(I:C) and CpG adsorb strongly to AH to form an effective combination adjuvant. This adjuvant demonstrated enhanced antigen uptake and activation of dendritic cells *in vitro* and induced a robust and balanced immune response in vivo. Moreover, this formulation avoided the systemic proinflammatory cytokine release associated with injection of soluble TLR agonists. We suggest that this combination adjuvant has utility for vaccines in which aluminum adjuvants alone do not provide adequate stimulation of the immune response.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.033.

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