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Aluminum Hydroxide And Aluminum Phosphate Adjuvants Elicit A Different Innate Immune Response

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ABSTRACT

Aluminum hydroxide ($Al(OH)_3$) and aluminum phosphate ($AlPO_4$) are widely used adjuvants in human vaccines. However, a rationale to choose one or the other is lacking since the differences between molecular mechanisms of action of these adjuvants are unknown. In the current study, we compared the innate immune response induced by both adjuvants *in vitro* and *in vivo*. Proteome analysis of human primary monocytes was used to determine the immunological pathways activated by these adjuvants. Subsequently, analysis of immune cells present at the site of injection and proteome analysis of the muscle tissue revealed the differentially regulated processes related to the innate immune response *in vivo*. Incubation with $Al(OH)_3$ specifically enhanced the activation of antigen processing and presentation pathways *in vitro*. *In vivo* experiments showed that only intramuscular (I.M.) immunization with $Al(OH)_3$ attracted neutrophils, while I.M. immunization with $AlPO_4$ attracted monocytes/macrophages to the site of injection. In addition, only I.M. immunization with $Al(OH)_3$ enhanced the process of hemostasis after 96 hours, possibly related to neutrophilic extracellular trap formation. Both adjuvants differentially regulated various immune system-related processes. The results show that $Al(OH)_3$ and $AlPO_4$ act differently on the innate immune system. We speculate that these different regulations affect the interaction with cells, due to the different physicochemical properties of both adjuvants.

Introduction

Most of the inactivated vaccines currently available require the use of an adjuvant to boost the immune response. Since the early 20th century, aluminum salts are known for their adjuvant activity.[1] Many vaccines contain aluminum hydroxide ($Al(OH)_3$) or aluminum phosphate ($AlPO_4$).[2] The immunological mechanisms of action attributed to aluminum salts are several:

1) *Depot effect*. Glenny *et al.* observed that the clearance of toxoids adsorbed to aluminum potassium sulfate was delayed compared to non-adsorbed toxoids *in vivo*;[3]
2) *Activation and maturation* of antigen presenting cells by both aluminum-based adjuvants occur both *in vivo* and *in vitro*, with and without antigen present;[4-5]
3) *Enhancement of the expression of chemotactic proteins in vitro*. Al$(OH)_3$ and $AlPO_4$ attract immune cells to the site of injection, *e.g.* inflammatory monocytes, dendritic cells, neutrophils, natural killer cells, eosinophils and $CD11^+$ cells, in the presence and absence of an antigen, *in vivo*;[4-9]
4) *Activation of the inflammasome*, inducing the secretion of inflammatory cytokine IL-1β, both *in vitro* and *in vivo*, upon $Al(OH)_3$ stimulation in the presence of an antigen;[10-11]
5) *Complement activation* in human sera (*in vitro*) upon $Al(OH)_3$ stimulation, in the presence of an antigen;[12]
6) *Release of Danger Associated Molecular Patterns* (DAMPs) upon cell death, induced by $Al(OH)_3$. DAMPs like uric acid and DNA can

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induce cell priming, inflammasome activation, IL-1β secretion, and MHC class II antigen presentation *in vitro* and *in vivo*, in the presence of an antigen;[13-15]

7) *Induction of a T helper (Th) type 2 response*, by inducing IL-4 secretion, *in vivo* and *in vitro*.[6,9]

Most of the molecular mechanisms described above were obtained in studies focused only on $Al(OH)_3$ and not on $AlPO_4$. These studies were mostly conducted with classical immunological techniques, such as flow cytometry, ELISA and multiplex immune assays (MIA). More recently, comprehensive, nonbiased approaches have emerged, *e.g.* genomics and proteomics.[16-17] However, the use of these techniques in adjuvant research is still limited.

In many vaccine formulations, either of the two adjuvants are used without a clear rationale of choosing between the two adjuvants. Sometimes the degree of antigen adsorption is used as a criterion. As $Al(OH)_3$ has a high Isoelectric Point (IEP), $Al(OH)_3$ often adsorbs antigens more efficiently, compared to $AlPO_4$, that has a neutral or slightly acidic IEP. However, the link between antigen adsorption in the formulation and immunogenicity does not always hold true.[18] Antigens may rapidly desorb from the adjuvant *in vivo*.[19] A link between adsorption and adjuvant activity is described, both positive and negative,[20-21] although it is not clear whether a link between the degree of adsorption and adjuvant effect is causal. There may be other effects than adsorption of antigen causing the adjuvant effect, *e.g.* interaction with Antigen Presenting Cells (APCs). Thus, knowledge on the immune-modulating effect of both adjuvants is important to substantiate the use of $Al(OH)_3$ versus $AlPO_4$ as an adjuvant.

In the current study, we compared the effects of $Al(OH)_3$ and $AlPO_4$ *in vitro* and *in vivo* by flow cytometric assays and quantitative mass spectrometry-based proteomics. These two adjuvants, are applied in many products of the major human and veterinary vaccine producers. The adjuvant effects were studied in human primary monocytes, since these prominent mononuclear phagocytes play an important role in bridging the innate and adaptive immune response.[22] In addition, we focused on the site of injection *in vivo* to study the initiation of the immune response in mice. The recruitment of immune cells to the site of injection was studied, as well as changes in the proteome of the site of injection. The results of this study show that both adjuvants attract different cell types to the site of injection and are different in inducing immune system-related processes.

Materials and Methods

Ethics Statement

The human monocyte study was conducted according to the principles expressed in the Declaration of Helsinki. All blood donors gave a written-informed consent before collection and use of their samples. All blood donations, provided by the Dutch National Institute for Public Health and the Environment (RIVM, Bilthoven; the Netherlands) were specifically donated for primary cell isolation. This research goal was explicitly approved by the accredited Medical Research Ethics Committee (MREC), METC, Noord-Holland in the Netherlands. All blood samples were processed anonymously.

Animal studies complied with the ARRIVE guideline and were approved by the central committee animal studies (CCD, The Hague; the Netherlands) following the procedures of European legislation guidelines (2010/63/EU and law for animal testing (WOD); the Netherlands). The specific experiments performed in this study were approved by the authority for animal welfare (IvD) and the Scientific committee (WTC) of Intravacc (Bilthoven; the Netherlands).

Reagents Used for Cell Incubation

$Al(OH)_3$ is Alhydrogel® 2% (Brenntag Biosector a former organization of Croda Denmark, Frederikssund; Denmark). $AlPO_4$ is AdjuPhos® (Brenntag Biosector, Frederikssund; Denmark). Lipopolysaccharide (LPS) from *E.coli* K12, referred to as LPS, was used as a positive control and was obtained from Invivogen (San Diego, CA; USA).

Primary Monocyte Culture and Adjuvant Stimulation

Fresh peripheral blood was obtained from four healthy volunteers and collected in heparin-coated tubes. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood using Ficoll density centrifugation at 1,000*xg* for 30 minutes. Monocytes were isolated from the PBMC fraction using positive selection by CD14 microbeads and magnetic LS MACS columns (Miltenyi Biotech, Bergisch Gladbach; Germany). A purity check was performed by flow cytometric analysis of CD14 cell surface expression.[23] Monocytes were ≥95% pure. The monocytes were cultured in a 24-well culture plate at a density of $0.6 \bullet 10^6$ cells/ml, 1 ml/well, in Roswell Park Memorial Institute 1640 (RPMI) (Gibco, Thermo Fisher Scientific, Waltham, MA; USA) supplemented with 10% Fetal Calf Serum (FCS) (Serana; Germany) and 100 units/ml penicillin, 100 μg/ml streptomycin and 0.3 μg/ml L-glutamine (P/S/G) (Invitrogen, Thermo Fisher Scientific, Waltham, MA; USA). Isolated monocytes were either left untreated or were incubated with $Al(OH)_3$ or $AlPO_4$ with a final concentration of 10 μg aluminum equivalents/ml at 37°C with 5% CO_2 for 24 or 48 hours. LPS (100 ng/ml) was used as a positive control.

Protein Isolation, Digestion and Labeling

To disrupt the cells and isolate the proteins from the cells, 500 μl of 4 M guanidine•HCl in 100 mM phosphate buffer pH 7.5 was added to the cell culture plates. The cell suspensions were incubated at 4°C for 30 minutes. During this incubation step, the cell suspensions were subjected to a freeze-thaw step. Lysed cells were stored at -80° C. After the cell lysis, a 50-μl aliquot of each sample was used to determine the protein concentration with the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Waltham, MA; USA) according to the manufacturer's protocol. To adjust the pH of the lysates and to reduce the concentration of guanidine•HCl to 1 M, the samples were diluted four times in 100 mM phosphate buffer pH 7.5. Next, the isolated proteins were digested with Lys-C (Roche, Basel; Switzerland) with an enzyme-to-substrate ratio of 1:10 for 4 hours at 37°C, after which fresh Lys-C was added (enzyme-to-substrate ratio 1:10) for an overnight incubation at 37°C. Samples were normalized on protein content, desalted using solid phase extraction on C18 SPE columns (Waters Corporation, Milford, MA; USA) according to the manufacturer's protocol and dried with centrifugation under reduced pressure, after which the samples were labeled per condition using Tandem Mass Tag labeling-10plex (TMT10plex) (Thermo Fisher Scientific, Waltham, MA; USA) according to the manufacturer's protocol. Samples were pooled and dried by centrifugation under reduced pressure. Next, the pooled samples were dissolved in double-distilled water (Milli-Q) water containing 5% dimethyl sulfoxide (DMSO) and 0.1% formic acid (FA) for mass spectrometry-based proteome analysis.

In Vivo Studies

Female (20 individuals) and male (30 individuals) BALB/c mice (age 6-8 weeks, specified pathogen free) were obtained from Charles River Laboratories. Mice were randomly divided in ten groups of five mice (three male and two female mice per group). The males and females were housed separately and per group in Macrolon II cages

with top filter. Three groups were injected intramuscularly (I.M.) in both quadricepses with $Al(OH)_3$ (100 μg aluminum equivalent), in 50 μl phosphate-buffered saline (PBS) pH 7.2 (Gibco, Thermo Fisher Scientific Waltham, MA; USA). Three groups were injected I.M. in both quadriceps with $AlPO_4$ (100 μg aluminum equivalent) in 50 μl PBS. Three control groups were injected with PBS only. One control group was not treated at all and euthanized after 96 hours. Injections were given under anesthesia (isoflurane/oxygen). Groups were euthanized and muscles were taken from individual mice 24, 48 and 96 hours after immunization and placed in All Protect (Qiagen, Venlo; the Netherlands). Animals with visual bite injuries were excluded from further sample processing, since these injuries might result in an inflammatory response, impacting the response to be measured.

Muscle Cell Isolation

During sample collection, the same part of each muscle was separated, sliced in small cubes, placed in Dulbecco's modified Eagle medium (DMEM) (Gibco, Thermo Fisher Scientific Waltham, MA; USA) and kept on ice. Samples were supplemented with Hanks-balanced salt solution (HBSS) and 0.2% collagenase B (Roche, Basel; Switzerland). Samples were rotated at 37°C for 30 minutes, after which the same amount of collagenase B was added and samples were again rotated for 30 minutes at 37°C. The remaining cell suspensions were filtered over a cell strainer (0.7 μm) and washed with DMEM containing 10% FCS and 1% P/S/G. Samples were frozen at -135°C in DMEM medium containing 40% FCS and 10% DMSO for subsequent analyses. Muscle cell isolations containing a low number of cells were excluded from further sample processing.

Muscle Protein Extraction and Labeling

Whole muscles were homogenized in 1 ml of all prep lysis buffer (Qiagen, Venlo; the Netherlands) with the fast prep-24 classic instrument: the samples were shaken 6 times 30 seconds at a speed of 6 meters/second (m/s), with cooling between each 30 seconds of shaking. Next, 500 μl sample was used for protein extraction with the all prep kit (Qiagen; Venlo; the Netherlands) according to the manufacturer's protocol. Protein pellets were reconstituted in 8 M urea (which was degassed with helium to prevent carbamylation of proteins) in 100 mM phosphate buffer pH 7.5. After reconstitution, urea concentrations were reduced to 1 M. A protein content analysis was performed using the BCA kit (Pierce Biotechnology, Waltham, MA; USA) according to the manufacturer's protocol. Samples were normalized on protein content and digested with proteinase Lys-C (Roche, Basel; Switzerland) in a 1:10 enzyme-to-substrate ratio at 37°C. After 4 hours, fresh Lys-C was added in a 1:10 enzyme-to-substrate-ratio for an overnight incubation at 37°C. The individual protein samples were labeled with TMT10plex (Thermo Fisher Scientific, Waltham, MA; USA) according to the manufacturer's protocol. Each TMT10plex contained the isolated proteins from the muscles of one mouse per group. Individual samples were pooled and dried with centrifugation under reduced pressure and a subsequent C18 solid phase extraction clean-up was performed, after which the samples were eluted with 90% acetonitrile (AcN) and 0.5% acetic acid in Milli-Q water. Samples were centrifuged under reduced pressure and reconstituted in Milli-Q water containing 5% DMSO and 0.1% FA for further analysis.

Flow Cytometry

The muscle samples frozen in medium were rapidly thawed at 37° C in a water bath. Subsequently, the samples were placed in a 15-ml tube with the addition of 4 ml medium (RPMI supplemented with 1% P/S/G and 10% FCS). Samples were washed with medium and subsequently with FACS buffer (PBS + 0.5% bovine serum albumin + 0.5 mM ethylenediaminetetraacetic acid (EDTA)). Samples were divided into two fractions. One of the fractions was left unstained and was used as a control. The other fraction was stained with AF488-conjugated Ly-6G (BioLegend clone 1A8), PE-conjugated CD115 (BD Biosciences clone T38-320), BV510-conjugated CD11b (BD Biosciences clone m1/70), and BV711-conjugated F4/80 (BD Biosciences clone T45-2342), all in a 1:50 dilution, and Live Dead fixable viability stain 780 (BD Biosciences) in a 1:2000 dilution in FACS buffer for 30 minutes at 4°C. The muscle cells were washed and fixated by resuspending in FACS buffer containing 1% paraformaldehyde. Data was acquired on a flow cytometer (Attune Next, Thermo Fisher Scientific, Waltham, MA; USA). For each staining, Fluorescence Minus One (FMO) controls were performed. Samples were compared to their unstained control and to the PBS control group at the corresponding time point. Data was analyzed using FlowJo software version 10 (Three Star).

LC MS/MS Analysis of Human Monocytes and Mouse Muscle Cells

Peptide separation was performed on an Agilent 1290 system (Santa Clara, California; USA). Peptides were trapped on a 2-cm L x 100-μm I.D. trapping column (Reprosil-Pur C18-AQ, df=5 μm; Dr. Maisch, Ammerbuch; Germany) and separation was performed on a 30-cm L x 50-μm I.D. analytical column (Reprosil-Pur C18-AQ, df=3 μm; Dr. Maisch, Ammerbuch; Germany), both packed in-house. Solvent A was 0.1% FA in Milli-Q water and solvent B was 0.1% FA in AcN (Biosolve, Valkenswaard; the Netherlands). The peptides were separated in 195 minutes in a nonlinear gradient (15 minutes at 0% B for peptide loading on the trapping column, subsequently followed by a gradient of 160 minutes from 0% to 30% B, a 15-minutes gradient to 45% B and 5 minutes at 65% B) optimized as described by Moruz *et al.*[24] The column effluent was electro-sprayed directly into the MS using a gold-coated fused silica tip of 3.5 μm tipID, with a spray voltage of 1.8 kV.

Mass spectrometric data was acquired on a Tribrid Orbitrap Fusion Lumos (Thermo Fisher Scientific, Waltham, MA; USA). The full scan (MS1) spectra were acquired with a scan mass range of 350-1,500 at 120,000 resolution (FWHM) with an Orbitrap readout. For the MS1, the automatic gain control (AGC) was set to 400,000 and the maximum injection time was 50 ms. Top speed mode was used with a duration of 3 s for the *in vitro* samples and 5 s for *in vivo* mouse muscle cells, where precursor ions were selected with an intensity >5,000 for fragmentation (MS2). Charge states between 2 and 7 were selected for MS2 and fragmentation was performed using Collision-Induced Dissociation (CID) in the linear ion trap (LTQ) with a normalized collision energy of 35%. In MS2, the AGC was set to 10,000 and the maximum injection time was 100 ms. Synchronous-precursor-selection was enabled to include up to 5 MS2 fragment ions in MS3. The fragment ions were further fragmented by higher energy collision dissociation with a normalized collision energy of 60%. The TMT reporter ions were analyzed in the Orbitrap analyzer, the AGC was set to 100,000 and the maximum injection time was set to 240 ms for the *in vitro* samples and 160 ms for the *in vivo* mouse muscles. Each individual sample was analyzed three times to optimize protein identification and quantification.

Analysis of Proteomics Data

Proteomics data was analyzed with Proteome Discoverer 2.1 (Thermo Fisher Scientific, Waltham, MA; USA) with default settings unless stated otherwise. Precursor mass tolerance was set to 5 ppm. MS2 scans were searched against the human Uniprot protein database from November 2014, containing 23,048 entries or the *Mus musculus* database, using the SequestHT search engine with a full enzyme

specificity for Lys-C as described previously.[23] The quantification node was used to obtain relative expression values, where TMT10plex was defined as the quantification method, with an integration tolerance of 0.2 Da. Cite percolator was used to filter the peptide to spectrum mass with a false discovery rate (FDR) of <5%.

Data was normalized by performing a median correction as described previously.[23] Data of three biological replicates for human samples and five biological replicates for mice samples were compared. Proteins that were upregulated or downregulated by 1.5 fold or more compared to the control (based on being >2.5x the median coefficient of variation (CV) as described previously[23] in at least two replicates (human monocyte samples) or a factor 2 in at least three out of five mice per group), were considered significantly regulated. This factor 2 was based on approximately 3x the median CV of 28%, analogous to our previous studies.[23] The regulated proteins were imported in Panther[25] to identify regulated pathways (FDR<0.05) within functional annotations provided by Gene Ontology (GO) biological processes.

Statistics

Flow cytometry data was analyzed using Graphpad Prism. Significance of difference was determined with a two-way analysis of variance (ANOVA) and a Tukey's test for multiple comparison correction.

Results

$AlPO_4$ and $Al(OH)_3$ Induce Different Pathways Related to Immune Activation in Human Primary Monocytes

Monocytes were incubated with $AlPO_4$ and $Al(OH)_3$ for 24 and 48 hours. Quantitative proteome analysis resulted in about 1,200 quantified proteins in all samples. After 24 hours, in both $AlPO_4$ and $Al(OH)_3$-incubated cells approximately 100 proteins were up- or downregulated compared to the expression in cells that were not incubated with adjuvant. These changes are reflected in the enrichment of various immune system-related pathways after incubation with both $AlPO_4$ and $Al(OH)_3$ (GO terms) (Fig. 1), including the known $Al(OH)_3$-related pathways *regulation of complement activation*[12] and *regulation of humoral immune response*. In addition, after 24 hours of incubation, the pathways *regulation of acute inflammatory response* and *immune response* were specifically upregulated in cells incubated with $Al(OH)_3$. The pathways specifically enriched in cells incubated with $AlPO_4$ were related to *viral transcription* and *viral gene expression*. After 24 hours of incubation with $AlPO_4$, the pathway *antigen processing and presentation* was downregulated, while this was not the case in $Al(OH)_3$-incubated monocytes.

After 48 hours of incubation, cells incubated with either one of the adjuvants showed upregulation of many immunologically relevant pathways, *e.g. (positive regulation of) defense response, immune response, viral process* and *blood coagulation. Antigen processing and presentation pathways* were also upregulated by both $Al(OH)_3$ and $AlPO_4$, although the upregulation was considerably more profound for $Al(OH)_3$ compared to $AlPO_4$ (Fig. 1). Upon $Al(OH)_3$ incubation, typical proteins related to antigen processing and presentation via both human leukocyte antigen (HLA) class I and class II, were upregulated. Proteins related to antigen presentation via HLA class I specifically regulated by incubation with $Al(OH)_3$, included various proteasomal subunits related to antigen processing and presentation, *e.g.* proteasome subunits alpha type-2 (PSMA2) and beta type-3 (PSMB3) and 26S proteasome non-ATPase regulatory subunits 3 and 13 (PSMD3 and PSMD13, respectively). Proteins specifically upregulated by incubation with $Al(OH)_3$, related to antigen presentation via HLA class II consisted of: cathepsin (CTS) L1, dynactin subunit 2 (DCTN2), cytoplasmic dynein 1 intermediate chain 2 (DYNC1i2) and legumain (LGMN) (Table S1). Pathways uniquely induced upon 48 hours of $Al(OH)_3$ incubation included: *positive regulation of type IIa hypersensitivity* and *positive regulation of adaptive immune response*. After 48 hours of incubation, *positive regulation of complement activation* was solely induced in cells incubated with $AlPO_4$ (Fig. 1, Table S2).

These data show that both adjuvants activate the immune system and immune system-related pathways in monocytes. Distinct differences in the quality and kinetics of the immunogenicity of both adjuvants were observed. Fig. 1 clearly shows that the *in vitro* immune response towards $Al(OH)_3$ is more pronounced compared to $AlPO_4$, particularly after prolonged incubation (48 hours). Another important difference was found with respect to antigen presentation and processing, which was downregulated after 24 hours of incubation with $AlPO_4$ (with no regulation upon $Al(OH)_3$ incubation at this time point), whereas this pathway was strongly upregulated after 48 hours of incubation with $Al(OH)_3$, and only a minimal induction for $AlPO_4$ was observed.

$Al(OH)_3$ but not $AlPO_4$ Attracts Neutrophils to the Site of Injection

To determine whether the differences identified *in vitro* could also be observed in the *in vivo* experiments, mice were intramuscularly injected with the adjuvants. The cell types present at the site of injection after administration at time points 24, 48 and 96 hours, were analyzed with flow cytometry (gating strategy and live/dead cell proportions are specified in Fig. S3 and Table S4, respectively). Twenty-four hours after administration of $Al(OH)_3$, no significant increase in any cell population was observed compared to the control group (PBS injected mice) (Fig. 2A and B), while administration of $AlPO_4$ slightly induced the attraction of neutrophils ($CD11b^+$ $F4/80^-$ $Ly6G^+$) at this time point (Fig. 2A). After both 48 and 96 hours, $Al(OH)_3$ significantly increased neutrophil influx ($CD11b^+$ $F4/80^-$ $Ly6G^+$) at the site of injection compared to the control group, while for $AlPO_4$ this neutrophil population was not significantly different from the control group anymore. After 96 hours, the influx of neutrophils induced by $Al(OH)_3$ was significantly higher compared to control and $AlPO_4$-administered mice (Fig. 2A). In addition, $Al(OH)_3$ significantly increased the monocyte/macrophage population at the site of injection after 96 hours (Fig. 2B). $AlPO_4$ significantly induced an increase of the monocyte/macrophage population ($CD11b^+$ $F4/80^+$ $Ly6G^-$) both after 48 and 96 hours (Fig. 2B). Thus, $AlPO_4$ and $Al(OH)_3$ attracted immune cells to the site of injection, though there is a difference in the kinetics of monocyte/macrophage attraction. Also, $Al(OH)_3$ attracted significant numbers of neutrophils after 48 and 96 hours, while $AlPO_4$ induced the attraction of neutrophils only after 24 hours.

Distinct protein expression at the site of injection after administration of $Al(OH)_3$ and $AlPO_4$

Twenty-four hours after administration of $Al(OH)_3$ or $AlPO_4$, protein analysis of mouse muscles showed a large overlap (67%) in upregulated proteins between the two adjuvants. Proteins that were highly upregulated in both stimulation conditions were S100-A8 and S100-A9 (Table S5), which are proteins related to the presence of neutrophils and monocytes.[26] The pathway (GO term) *neutrophil aggregation* (attraction of neutrophils) was enriched for both incubation conditions (Fig. 3). Moreover, *(antibacterial) humoral immune response, hemostasis* and the related GO process *negative regulation* (*i.e.* inhibition) *of hemostasis* were also enriched. In addition, the processes *monocyte chemotaxis* and *acute phase response* were specifically enhanced by $Al(OH)_3$ (Fig. 3, extracted from Table S6). $AlPO_4$ uniquely induced the pathway *localization*, which is related to the movement of *e.g.* proteins, macromolecules and organelles in the cell, after 24 hours of stimulation (Fig. 3).

pathway information	24 hours		48 hours	
Immunological pathways	$Al(OH)_3$	$AlPO_4$	$Al(OH)_3$	$AlPO_4$
positive regulation of defense response			3	
defense response				1
immune system process	1		3	3
immune response	1		3	3
activation of innate immune response			3	
positive regulation of adaptive immune response			1	
innate immune response-activating signal transduction			3	
regulation of acute inflammatory response	1		1	2
regulation of immune effector process			1	2
viral transcription		1	3	
viral process			3	1
viral gene expression		1	2	
humoral immune response			-1	
regulation of humoral immune response	1	2	1	2
regulation of immune response			3	2
antigen processing and presentation		-1	3	1
antigen processing and presentation of exogenous peptide antigen via MHC class I			3	
antigen processing and presentation of exogenous peptide antigen via MHC class II			3	1
blood coagulation		2	3	3
regulation of blood coagulation	3	3	2	3
regulation of complement activation	1	1		2
positive regulation of complement activation				1
response to external stimulus				
regulation of wound healing	3	2	1	3
platelet aggregation			-3	
platelet degranulation	3	3	3	3
response to stress			3	2
response to toxic substance			1	
response to organic substance			3	
response to chemical			3	1
transport-related pathways				
localization	2	3	3	3
cellular localization			3	
transport	2	3	3	3
vesicle-mediated transport	3	3	3	3
endocytosis			2	3
positive regulation of phagocytosis			1	
exocytosis	3	3	3	3
secretion	3	3	3	3
biological regulation-related pathways				
biological regulation	1	2	3	2
small molecule metabolic process			3	
negative regulation of metabolic process		1	3	
protein catabolic process			3	
regulation of catabolic process			3	
negative regulation of catalytic activity	3	3	3	3
cellular macromolecule catabolic process			3	
homeostasis-related pathways				
cellular component organization	1	2	3	3
positive regulation of type Iia hypersensitivity			1	
DNA packaging			-3	
negative regulation of apoptotic process				
macromolecule complex assembly				
mRNA splicing, via spliceosome			-2	
mRNA processing			-1	
mRNA metabolic process		1	-1	

Figure 1. Heatmap of regulated processes in human monocytes. Red and green colors depict up and down regulation, respectively. For each incubation condition, a summary of the enriched pathways is depicted. The pathways are grouped based on immunological and homeostatic features. The intensity of the color and the numbers correspond to the significance of the pathway: (-)1, (-)2 and (-)3 correspond to a p-value of <0.05, <0.01 and <0.001, respectively.

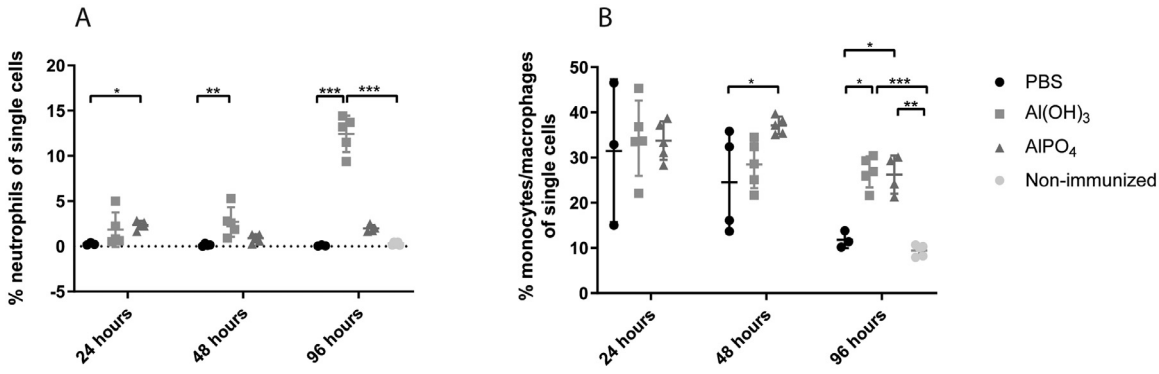

Figure 2. Cell populations at the site of injection in mice muscles. Percentage of neutrophils (A) and percentage of macrophages/monocytes (B) of single cells isolated from muscle. Significance of difference was determined with a two-way ANOVA with Tukey's testing for multiple comparison, p-values <0.05 are indicated with an *, p-values <0.01 are indicated with ** and p-values <0.001 are indicated with ***.

Forty-eight hours after injection, the overlap between the upregulated proteins by $Al(OH)_3$ and $AlPO_4$ at the site of injection was 62%. Both adjuvants still enhanced *neutrophil aggregation, hemostasis* and the GO-related term *negative regulation* (*i.e.* inhibition) *of hemostasis*. In addition, various immune system-related processes, *e.g. adaptive immune response* and *innate immune response* were enriched (Fig. 3). Moreover, $Al(OH)_3$ specifically induced various pathways, such as *regulation of cell death* and re*gulation of interleukin-8 production*. IL-8 is one of the main neutrophil chemoattractant molecules. Its upregulation is thus in accordance with the detection of neutrophils at the injection site in response to $Al(OH)_3$ only. $AlPO_4$ did not regulate any additional immunological pathways compared to pathways regulated by $Al(OH)_3$.

Ninety-six hours after injection, the overlap in the upregulated proteins in mice muscles between $Al(OH)_3$ and $AlPO_4$ was 57%. Both adjuvants induced immune system-related processes, *e.g. innate immune response, (antimicrobial) humoral response, hemostasis* and *defense response*. In contrast to *leukocyte aggregation*, which was upregulated by both adjuvants at all time points, $Al(OH)_3$ uniquely induced *neutrophil chemotaxis* and *neutrophil migration*, showing that pathways related to neutrophil influx are specifically upregulated by $Al(OH)_3$ (Fig. 3). This is in agreement with the flow cytometry data that shows the presence of a large population of neutrophils upon $Al(OH)_3$ stimulation (Fig. 2).

Other immunological processes that were upregulated after injection with $Al(OH)_3$ were negative regulation or inhibition processes, *e.g. negative regulation of immune system process*, which could either represent tolerance to the adjuvant or the contraction phase after initiation. $AlPO_4$ specifically enhanced *positive regulation of cell-cell adhesion*, the *inflammatory response, adaptive immune response* and *negative regulation* (inhibition) *of hemostasis* (Fig. 3).

These data prove that the innate immune response induced by both aluminum-based adjuvants *in vivo* is quite similar at 24 hours after injection. However, after 96 hours differences were observed between the response in $Al(OH)_3$ and $AlPO_4$ injected muscles which included a strong induction of neutrophil-related pathways in $Al(OH)_3$-injected muscles, which was considerably less prominent after $AlPO_4$ injection. In addition, there was a difference in the processes related to hemostasis after 96 hours: both adjuvants induced and inhibited the processes of hemostasis, with the exception of the 96-hour time point in which $Al(OH)_3$ only induced hemostasis.

Discussion

Aluminum-containing adjuvants are often used in human vaccines. Although the mechanisms of action have been extensively studied in recent years, comparison of the molecular responses to $Al(OH)_3$ *versus* $AlPO_4$ has not been performed. This study reveals that both $Al(OH)_3$ and $AlPO_4$ activate innate immune responses, briefly summarized in Table 1. Two main differences were observed: (*i*) antigen processing and presentation pathways in monocyte cultures were mainly upregulated by $Al(OH)_3$ and (*ii*) $Al(OH)_3$ attracted more neutrophils to the site of injection *in vivo*, possibly related to neutrophil extracellular trap (NET) formation and hemostasis.

Activated monocytes play a fundamental role in antigen processing and presentation.[22] A clear difference was observed in the activation of antigen processing and presentation pathways between $Al(OH)_3$ and $AlPO_4$: incubation with $Al(OH)_3$ strongly activated the pathway of *antigen processing and presentation* in monocytes, both via HLA class I and HLA class II, as described previously,[14,17,23] while incubation with $AlPO_4$ only induced a limited response. This may be potentially related to the differences in physicochemical properties or biological activity between $Al(OH)_3$ and $AlPO_4$.[27] This enhanced uptake is most likely related to the physicochemical properties of the adjuvants, particularly charge and size. With respect to charge, $Al(OH)_3$ is positively charged at a physiological pH, even though this charge may be partly shielded by proteins and salts in the culture medium.[28] This positive charge of $Al(OH)_3$ allows for stronger interactions with the negatively charged cell membrane, compared to the slightly negatively charged $AlPO_4$.[29] With respect to size, $Al(OH)_3$ particles are expected to have a more suitable size (*i.e.* smaller-sized) for phagocytosis by monocytes compared to $AlPO_4$ which has been suggested by Mold *et al.*[30] In the current study, this was observed as well, since only $Al(OH)_3$ activated phagocytosis. Thus, both size and charge probably lead to an increased uptake of $Al(OH)_3$ and enhanced antigen processing and presentation as well as related pathways, *e.g. activation of immune response* and *innate immune response-activating signal transduction*.

The attraction of neutrophils to the site of injection was affected by $Al(OH)_3$ administration after 48 (limited) and 96 hours (extensively) and only to a small extend after 24 hours of $AlPO_4$ stimulation. In agreement with this, injection with $Al(OH)_3$ uniquely induced *neutrophil chemotaxis* and *neutrophil migration* after 96 hours. Neutrophils are capable of eliciting an inflammatory response by binding and ingesting bacteria when activated.[31] In addition, neutrophil presence can result in the formation of NETs. Extracellular traps (ETs) are networks of chromatin and histones, which are extruded from immune cells. NETs are specifically extruded from neutrophils and can trap and kill bacteria[32] as well as further amplify neutrophil recruitment induced in the presence of $Al(OH)_3$.[33] It was previously described that neutrophils contribute to the adjuvant effect of $Al(OH)_3$ both alive and dead due to netosis, by inducing antigen-specific T cell expansion, B cell differentiation and B cell class switching in the presence of an antigen.[33] Mice deficient in netosis had significantly

Pathway	$Al(OH)_3$ 24 hrs up	$AlPO_4$ 24 hrs up	$Al(OH)_3$ 48 hrs up	$AlPO_4$ 48 hrs up	$Al(OH)_3$ 96 hrs up	$AlPO_4$ 96 hrs up
chemotaxis-related pathways						
neutrophil aggregation (GO:0070488)	2	2	3	2	2	2
neutrophil migration (GO:1990266)	2	1			1	
neutrophil chemotaxis (GO:0030593)	2	1			2	
mononuclear cell migration (GO:0071674)	1					
monocyte chemotaxis (GO:0002548)	1					
leukocyte cell-cell adhesion (GO:0007159)	2	2	2	1	2	2
leukocyte aggregation (GO:0070486)	2	1	2	2	1	1
leukocyte migration involved in inflammatory response (GO:0002523)	1	1	2	1	1	1
leukocyte chemotaxis (GO:0030595)	2	2	2	1	2	
granulocyte chemotaxis (GO:0071621)	3	3	2	2	2	1
positive regulation of cell-cell adhesion (GO:0022409)	2	1	2	2		1
chemotaxis (GO:0006935)	2	1	1	1	1	
immune system-related pathways						
regulation of cytokine secretion involved in immune response (GO:0002739)	1	1	2	2	1	1
negative regulation of cytokine production involved in immune response (GO:0002719)	1	1	1	1	1	1
regulation of cytokine secretion (GO:0050707)	1		1	1		
regulation of interleukin-8 production (GO:0032677)			1			
immune response (GO:0006955)	2	3	3	2	2	2
innate immune response (GO:0045087)	3	3	3	3	3	3
inflammatory response (GO:0006954)	2	2	2	2		2
acute-phase response (GO:0006953)	1					
adaptive immune response (GO:0002250)	1	1	2	2		1
negative regulation of immune system process (GO:0002683)	1				1	
negative regulation of immune effector process (GO:0002698)	2	2	2	1	2	
antimicrobial humoral response (GO:0019730)	2	2	2	2	2	2
induction of bacterial agglutination (GO:0043152)	2	2	2	2	2	2
antibacterial humoral response (GO:0019731)	1		1	1	1	
humoral immune response (GO:0006959)		2	2	2		1
defense response (GO:0006952)	3	3	3	3	2	3
wouding and chromatin procssing pathwyas						
hemostasis (GO:0007599)	2	3	3	3	2	3
negative regulation of hemostasis (GO:1900047)	2	2	2	2		2
blood coagulation (GO:0007596)	2	3	3	3	2	3
wound healing (GO:0042060)	2	3	3	3	2	2
response to wounding (GO:0009611)	2	3	3	3	2	2
blood coagulation, fibrin clot formation (GO:0072378)	3	3	3	3	2	3
regulation of blood coagulation (GO:0030193)	3	3	3	3	2	3
chromatin assembly (GO:0031497)					1	
chromatin assembly or disassembly (GO:0006333)					1	
cell death-related pathways						
regulation of cell death (GO:0010941)	1	1	1			
regulation of programmed cell death (GO:0043067)	2	1	1	1		
negative regulation of extrinsic apoptotic signaling pathway (GO:2001237)	3	2	2	2	1	1
regulation of extrinsic apoptotic signaling pathway (GO:2001236)	2	2	1	1		1
negative regulation of apoptotic signaling pathway (GO:2001234)	2	1	1	1		
homeostasis-related pathways						
localization (GO:0051179)		1	1			
neutral lipid metabolic process (GO:0006638)	2	2	3		1	1
cholesterol metabolic process (GO:0008203)	1	2	3		1	1
lipoprotein metabolic process (GO:0042157)	1	2	3		1	1
positive regulation of metabolic process (GO:0009893)			2	1		1
neutral lipid catabolic process (GO:0046461)	3	2	3	1	2	2
positive regulation of lipid catabolic process (GO:0050996)	3	2	3	1	2	2

Figure 3. Pathway analysis in mouse muscle cells. A subset of pathways extracted from Supplementary S6 that were upregulated in muscles injected with either Al(OH)3 or AlPO4 relative to PBS-injected controls at 24, 48 and 96 hours post injection. The numbers 1-3 indicate the significance of the regulations with an FDR of <0.05, <0.01 and <0.001, respectively. Empty cells indicate that there was no regulation of the specific pathway for that condition.

less antigen specific T cells and germinal B cell centers.[33] Based on these data, it was concluded that trap formation indeed contributes to the adjuvant effect of $Al(OH)_3$ upon vaccination.[33] In our current study, only a small increase in neutrophils at the injection site was observed 24 hours after $AlPO_4$ administration, which did not coincide with an upregulation of proteins related to NET formation. This implies that $AlPO_4$ probably does not induce NET formation, or only to a very limited extent. However, the formation of nodules that resemble ETs was described previously for $AlPO_4$.[34] These ETs did not depend on the presence of neutrophils, but on the presence

Table 1
Main differences between $Al(OH)_3$ and $AlPO_4$. $Al(OH)_3$ is equally or more active in pathway induction compared to $AlPO_4$. No pathways were detected that were more upregulated by $AlPO_4$ as compared to $Al(OH)_3$.

Adjuvant efficacy comparison	Effect or pathway
$Al(OH)_3 > AlPO_4$	Antigen processing Neutrophil attraction Lipid metabolism
$Al(OH)_3 = AlPO_4$	Transport/secretion Pathways related to wounding
$Al(OH)_3 < AlPO_4$	None

of fibrinogen (FETs) and were induced by both $Al(OH)_3$ and $AlPO_4$.[34] However, these FETs were observed after intraperitoneal administration of the adjuvants. Via this route, the adjuvants are exposed to many types of immune cells which are also able to form ETs[33,35-36] implying that the route of administration may have an effect on ET formation.

The formation of NETs and FETs is related to the processes of *hemostasis*[33-34,37-38]. Ninety-six hours after I.M. injection with either $Al(OH)_3$ or $AlPO_4$, the process of hemostasis was upregulated in mouse muscle cells. However, only for $Al(OH)_3$ this coincided with the presence of a significant population of neutrophils and the enrichment of histone 1.3 and 2A, proteins that are often found in NETs.[34] Finally, also the pathways *neutrophil chemotaxis* and *neutrophil migration* were upregulated by $Al(OH)_3$ and $AlPO_4$ (24 hours) and $Al(OH)_3$ (96 hours). The obtained data for the 96-hour time point of the $Al(OH)_3$-administered group, could indicate that NET formation might occur upon $Al(OH)_3$ stimulation, which is in agreement with the NET formation that was found previously.[33] Other proteins found in (F)ETs were upregulated by both adjuvants, *e.g.* fibrinogen alpha chain and histone 4 (Table S5). This upregulation of proteins associated with (F)ETs, upon both $Al(OH)_3$ and $AlPO_4$ stimulation, implies that (F)ET formation might also occur upon I.M. injection of $Al(OH)_3$ and $AlPO_4$.

Attraction of a large population of neutrophils to the site of injection and the activation of more immunological pathways by $Al(OH)_3$ implies that this is perhaps a more pro-inflammatory adjuvant compared to $AlPO_4$. This might result in more side effects since neutrophils also contain high levels of cytotoxic compounds, often associated with tissue inflammation and potential side effects.[31] Whether this results in a stronger adjuvant effect is not clear. Studies comparing the adjuvant effect of $Al(OH)_3$ or $AlPO_4$, *i.e.* in the presence of antigen, have been contradicting. Either no differences between the antibody titers were reported[29,39-40] or a trend towards a stronger response towards antigen adsorbed to $AlPO_4$[29] or a better response when $Al(OH)_3$ was used as an adjuvant.[20] The stronger response towards $Al(OH)_3$ was associated with a higher adsorption of the antigen to $Al(OH)_3$.[20] Other potential explanations might be: intrinsic immunogenicity of the adjuvant, as suggested by Berthold *et al.*; antigen-adjuvant ratio; and the dose of both the adjuvant and the antigen.[40] Finally, the ability of the antigen to desorb from the adjuvant can also be involved. If the interaction is too strong, the adjuvant effect can be negatively influenced as reviewed by Clapp *et al.*[18]

A major difference between the mechanisms identified *in vitro* and *in vivo* is that, unlike the *in vitro* data, indications for antigen processing and presentation were not identified in the *in vivo* data. The reason for this difference is not known. $Al(OH)_3$ may be coated by serum proteins present in the culture medium, which might result in the cell entrance of adjuvant-serum protein complexes during *in vitro* experiments. This might not happen *in vivo* upon $Al(OH)_3$ I.M. injection, since interstitial fluid is lower in protein content compared to serum.[41-42] In addition, proteins in culture medium might be identified as non-self-proteins (enhanced antigen presentation), while proteins in interstitial fluid are self-proteins. However, enhanced antigen processing and presentation upon $Al(OH)_3$ I.M. injection was described previously.[17] The reason that this is not identified in the current study could be that either different outcome parameters are used: mRNA *versus* protein, the *in vivo* expression of antigen processing-related proteins might occur predominantly at different locations, *e.g.* draining lymph nodes, or the presence of other APCs than only monocytes.

Some known mechanisms of action of aluminum salt-based adjuvants were confirmed in this study, *e.g. in vitro* upregulation of *complement activation* and *cell death* by $Al(OH)_3$[12-14] and recruitment of immune cells to the site of injection by both $Al(OH)_3$ and $AlPO_4$.[8-9,43-44] In addition, differences between the innate immune response after incubation of monocytes with $Al(OH)_3$ or $AlPO_4$ were observed *in vitro*. These *in vitro* differences were mainly quantitative since both adjuvants activated similar pathways, *e.g.* antigen presentation, although the level of activation was different. It needs to be taken into account that we have analyzed the cellular proteins and not the secreted proteins. In addition, some processes related to $Al(OH)_3$ adjuvant activity require the presence of an antigen or other additional components, such as activation of the inflammasome.[45] Monocytes were selected for our *in vitro* study, because of the fundamental role they play in antigen processing and presentation.[22] The identity of this cell type has been confirmed prior to the incubation by a CD14 gating strategy as described previously,[23] though it cannot be excluded that, upon stimulation, these monocytes differentiated towards either macrophages or dendritic cells.

The current study demonstrates that two commercially available aluminum salt-based adjuvants, $Al(OH)_3$ and $AlPO_4$, have a very distinct impact on the innate immune response both *in vitro* on human primary monocytes as well as *in vivo* in mice. Although the adjuvants are very frequently used by the major human and veterinary vaccine producers, similar type of adjuvants are sometimes also prepared *in situ*. These adjuvants, however, may have different properties than the commercially available forms of $Al(OH)_3$ and $AlPO_4$. Based on current data, $Al(OH)_3$ induces a more profound immunogenic response compared to $AlPO_4$, since $Al(OH)_3$ upregulated antigen processing and presentation pathways more strongly than $AlPO_4$. Additionally, considerably more immune system-related pathways were induced by $Al(OH)_3$ and, *in vivo*, more neutrophils were attracted to the site of injection as compared to $AlPO_4$.

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.01.014.

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