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T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection

Karen Smith Korsholm, Rune V. Petersen, Else Marie Agger and Peter Andersen

Department of Infectious Disease Immunology, Statens Serum Institute, Copenhagen, Denmark

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Correspondence: Dr K. S. Korsholm, Department of Infectious Disease Immunology, Vaccine Delivery & Formulation, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen, Denmark.
Email: kkn@ssi.dk
Senior author: Dr P. Andersen, email: pa@ssi.dk

Summary

Vaccine adjuvants activate the innate immune system and thus influence subsequent adaptive T-cell responses. However, little is known about the initial immune mechanisms preceding the adjuvant-induced differentiation of T-helper (Th) cells. The effect of a T-helper 1 (Th1) adjuvant, dimethyldioctadecylammonium liposomes with monophosphoryl lipid-A (DDA/MPL), and a T-helper 2 adjuvant, aluminium hydroxide [$Al(OH)_3$], on early, innate chemotactic signals and inflammatory cell influx at the site of injection was therefore investigated. Injection of the adjuvants into the peritoneal cavity of mice demonstrated distinct differences in the magnitude, quality and kinetics of the response. The inflammatory response to DDA/MPL was prominent, inducing high local levels of pro-inflammatory cytokines, chemokines and a pronounced inflammatory exudate consisting of neutrophils, monocytes/macrophages and activated natural killer cells. This was in contrast to the response induced by $Al(OH)_3$, which, although sharing some of the early chemokine signals, was more moderate and consisted almost exclusively of neutrophils and eosinophils. Notably, $Al(OH)_3$ specifically induced the release of a significant amount of interleukin (IL)-5, whereas DDA/MPL induced high amounts of tumour necrosis factor-α (TNF-α), IL-1α and IL-6. Finally, a microarray analysis confirmed that the effect of DDA/MPL was broader with more than five times as many genes being specifically up-regulated after injection of DDA/MPL compared with $Al(OH)_3$. Thus, the adjuvants induced qualitatively distinct local inflammatory signals early after injection.

Keywords: adjuvants; cytokines; inflammation; T-helper 1/T-helper 2

Introduction

With the vast amount of information on T-cell differentiation that has been obtained in recent years, and in particular that on the role of pathogen-associated molecular patterns (PAMPs) in the induction of immune responses, it should in theory be possible to tailor vaccines and adjuvants to selectively induce cell-mediated/humoral and T-helper 1/T-helper 2 (Th1/Th2) responses. This would obviously have tremendous impact on the ongoing

Abbreviations: $Al(OH)_3$, aluminium hydroxide; APC, antigen-presenting cell; CBA, cytometric bead array; CCL, C–C chemokine ligand; CXCL, C-X-C chemokine ligand; CXCR, C-X-C chemokine receptor; DC, dendritic cell; DDA, dimethyldioctadecylammonium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; IP, IFN-γ-inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; MPL, monophosphoryl lipid-A; NK, natural killer; PAMP, pathogen-associated molecular pattern; PE, phycoerythrin; PEC, peritoneal exudate cell; PRR, pattern-recognition receptor; RANTES, regulated on activation normal T-cell expressed and secreted; SSC, side scatter; Th, T helper; TLR, Toll-like receptor; TNF, tumour necrosis factor.

attempts to develop vaccines against some of the remaining challenges, such as human immunodeficiency virus (HIV), tuberculosis, chlamydia and hepatitis C. The common denominator for these infectious diseases is the fact that immunity resides largely within the cellular arm of the immune response, in contrast to infections for which efficient vaccines have already been developed, such as polio, diphtheria and measles, where immunity primarily depends on antibodies.

In practical vaccine development it has, however, proven difficult to translate the results obtained from defined *in vitro* observations to the complexity of the integrated immune system of living organisms, and limited information is currently available on the activity of aluminium salts, the only adjuvant in general use for human vaccines. In this regard, the early, innate inflammatory response induced by different adjuvants is a parameter that immunologists have only recently started to dissect. The inflammatory response is rapid and antigen-independent and it precedes the initiation of the antigen-specific response. It is characterized by the local release of pro-inflammatory cytokines, chemokines and the immediate influx of polymorphonuclear granulocytes from the bloodstream. Initiation of inflammation can occur through the activation of pattern-recognition receptors (PRRs), which are expressed in different forms and compositions by a variety of cells, including lymphocytes, granulocytes and endothelial cells.[1,2] Ligation of PAMPs to PRRs expressed by dendritic cells (DCs), the type of PRR and the surrounding cytokine milieu are all factors that contribute to DC maturation and hence influence the cytokines and costimulatory molecules expressed by the mature DCs.[3,4] As T-cell differentiation is influenced by the different costimulatory signals received from the DCs,[5] the early stimulation of this subset and the degree of inflammation at the site of injection are involved in shaping the ensuing immune response and thus have a major impact on the profile of the subsequent T-cell response.[6]

One adjuvant that induces strong Th1 responses in various animal models is monophosphoryl lipid-A (MPL) formulated with cationic dimethyldioctadecylammonium (DDA) liposomes (DDA/MPL).[7–9] Much is already known about the ability of this adjuvant to enhance antigen uptake, antigen presentation to T cells[10] and stimulate DCs through Toll-like receptors (TLRs),[11,12] but limited information exists on the mechanism of this adjuvant *in vivo*. By comparison, the mechanism operating when aluminium salts induce an immune response has until recently remained elusive and even in defined *in vitro* models its activity is less clear than that of DDA/MPL. Initially, aluminium salts were believed to work primarily through the formation of a depot of antigen at the site of injection. However, although aluminium compounds are able to adsorb a variety of vaccine antigens, and some aluminium compounds do remain at the site of injection for prolonged periods of time,[13] the actual importance of this mechanism has become unclear since it was shown that removing the injection site did not compromise the efficacy of an aluminium-adjuvanted vaccine.[14] In a recent study, the aluminium-containing Imject® (Pierce Biochemicals) adjuvant recruited and activated inflammatory monocytes to the site of injection through the induction of uric acid,[15] and in the presence of TLR agonists aluminium induces inflammasome activation.[16–18] Aluminium salts induce Th2 differentiation and humoral immunity,[19,20] but the exact mechanism, as well as the dependence on the classical Th2-inducing cytokine, interleukin (IL)-4, remains unclear both *in vitro*[21–25] and *in vivo*.[26,27] In this respect it is a problem that negative results are rarely published because this may lead to an erroneous bias in the general view of how aluminium salts function as adjuvants.

Here, we have investigated the early, innate chemotactic signals and inflammatory influx induced by Th1- and Th2-polarizing adjuvants. Using DDA/MPL as our model Th1 adjuvant and aluminium hydroxide [$Al(OH)_3$] as our model Th2 adjuvant, we have found that whereas the Th1 adjuvant induced high amounts of a large range of pro-inflammatory mediators and the influx of various cell types, the Th2 adjuvant induced a more discrete response with the involvement of only few mediators and a different inflammatory cell recruitment. The only Th2-specific signals found at the site of injection were an influx of eosinophilic granulocytes and the local release of IL-5.

Materials and methods

Animals and antibodies

Female BALB/c or C57BL/6 mice were purchased from Harlan (Horst, the Netherlands). The mice were allowed free access to water and food, and they were under 6 months of age when used in the experiments. The protocol for the animal experiments was approved by the Danish Council for Animal Experiments. Unless stated otherwise, BALB/c mice were used in the experiments. All antibodies, cytometric bead array (CBA) kits and enzyme-linked immunosorbent assay (ELISA) sets were purchased from BD Biosciences/Pharmingen (Brøndby, Denmark) unless otherwise stated. The following antibodies were used for flow cytometry: rat α-mouse F4/80-conjugated phycoerythrin (PE) (AbD Serotec, Hamar, Norway), rat α-mouse F4/80-conjugated biotin (Caltag Laboratories, San Fransisco, CA), purified rat α-mouse CD16/CD32, rat α-mouse CD11c–PE–Cy7/allophycocyanin/PE, rat α-mouse CD19–allophycocyanin/peridinin chlorphyll protein (PerCP)–Cy5.5, rat α-mouse CD3–allophycocyanin/fluorescein isothiocyanate (FITC), rat α-mouse CD49b–allophycocyanin/PE, rat α-mouse CD4–PE, rat α-mouse Ly-6G–PE, or streptavidin–PerCP–Cy5.5.

Medium

The medium used in the experiments was complete RPMI, consisting of the following: RPMI-1640 (Gibco Invitrogen, Taastrup, Denmark) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5×10^{-6} M β-mercaptoethanol, 1% (v/v) penicillin–streptomycin, 1% sodium pyruvate, 1 mM L-glutamine, 10 mM HEPES and 100 μg/ml of gentamicin (Gibco Invitrogen).

Preparation of DDA liposomes and vaccines

DDA liposomes were prepared using the aqueous heat method as follows. DDA (as a bromide salt; Avanti Polar Lipids, Alabaster, AL) was suspended in sterile distilled water at 2·5 mg/ml and heated to 80° for 20 min. The suspension was vortexed regularly during the heating period. Before injection, vaccine and adjuvant formulations were mixed and left at 21° for 30 min with intermittent mixing.

Animal experiments

Antigen-specific responses: C57BL/6 or BALB/c mice were immunized subcutaneously three times, at 2-week intervals, with 200 µl of sterile saline containing 2 µg of recombinant Ag85B–ESAT-6 (Statens Serum Institut, Copenhagen, Denmark) adjuvanted in 25 µg of detoxified MPL (Avanti Polar Lipids) and 250 µg of DDA liposomes (DDA/MPL) or in 500 µg of $Al(OH)_3$ (as 2% Alhydrogel; Brenntag Biosector, Frederikssund, Denmark). One week after the final immunization, six mice per group were bled. Single-cell suspensions of lymphocytes were washed and cultured in complete RPMI in round-bottom 96-well plates, containing 2×10^5 cells/well, and restimulated with 5 µg/ml of Ag85B–ESAT-6. After 3 days, the supernatants were collected and the cytokine release was analyzed by ELISAs. Four months after the final immunization, between three and six mice per group were bled and the serum was analyzed for Ag85B–ESAT-6-specific antibodies.

Adjuvant-specific responses: BALB/c mice were injected intraperitoneally (i.p.) with 200 µl of sterile saline containing either 25 µg of MPL and 250 µg of DDA liposomes or 500 of µg $Al(OH)_3$. Mice were killed at the indicated time-points after injection and the peritoneal exudate cells (PECs) were harvested by washing the peritoneal cavity as follows: 2 ml of cold RPMI containing 10% FBS was injected, the abdomen was massaged gently for 30 seconds and 1 ml was extracted. The cells were put on ice immediately. A small volume of medium was used to prevent excessive dilution of the peritoneal exudate. The cells were spun down, washed and stained for flow cytometry. The peritoneal lavage fluid (i.e. the first supernatant) was analyzed for cytokines/chemokines by ELISA and CBA. For use in the microarray analysis, cells were centrifuged and resuspended in RNAlater RNA stabilizing Reagent (Qiagen, Copenhagen, Denmark) immediately after extraction from the peritoneal cavity. The observed effect of the adjuvants was not caused by the injection procedure and related tissue injury because injection of physiological saline did not induce cell recruitment (data not shown).

Flow cytometry

PECs were washed and Fc receptors were blocked with α-mouse CD16/CD32. The cells were kept at 4° during staining. TO-PRO-3 iodide (Invitrogen/Molecular Probes, Taastrup, Denmark) was added at 100 nM immediately before acquisition on a FACSCanto (BD Biosciences, Brøndby, Denmark) to exclude dead cells. All analyses were performed using the FCS EXPRESS Software v3 (De-Novo Software, Thornhill, Canada) or FLOWJO FLOW CYTOMETRY Analysis Software (Tree Star Inc., Ashland, OR). Cell types were identified according to the following markers: B cells ($CD19^+$), DCs [$CD11c^+$ major histocompatibility complex class II (MHC class II)$^+$ $F4/80^-$ Ly-$6G^{-/lo}$], eosinophils (Ly-$6G^{int}$ $CD49b^+$ $CD3^-$ [side-scatter $(SSC)^{int/hi}$)], macrophages ($F4/80^+$ MHC class II^+ $CD11c^-$ Ly-$6G^{-/lo}$), monocytes ($F4/80^+$ MHC class II^- $CD11c^-$ Ly-$6G^{-/lo}$), neutrophils (Ly-$6G^{hi}$), natural killer (NK) cells [$CD49b^+$ $CD3^-$ (SSC^{lo})] and T cells ($CD3^+$ $CD49b^-$). For sorting of PECs from mice injected with $Al(OH)_3$ according to their forward-scatter (FSC)-SSC profile, a BD FACSCalibur (BD Biosciences) was used. At least 10 000 cells of each population were collected.

Differential staining

Total PEC and sorted populations were spun onto microscopy slides using a Shandon Cytospin® (Thermo Fisher Scientific, Slangerup, Denmark). The cells were subsequently stained with Hemacolor (Merck, Hellerup, Denmark) according to the manufacturer's protocol. The morphology of the cells was assessed using light microscopy.

Cytokine detection

Unless otherwise indicated, all cytokine analyses were performed using CBA. The peritoneal lavage fluid and supernatants were analyzed for C-C chemokine ligand (CCL)2 [monocyte-chemoattractant protein-1 (MCP-1)], IL-6, IL-10, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-12p70 using the CBA Inflammation kit according to the manufacturer's protocol. IL-5 was measured by ELISA using the IL-5 minikit (Endogen; Thermo Fisher, Rockford, IL) following the manufacturer's instructions. IL-1α was measured by ELISA using the IL-1α OptEia set, according to the protocol suggested by the manufacturer. CCL11 (Eotaxin-1) and CCL24 (Eotaxin-2) were measured by ELISA using the corresponding Mouse

DuoSets (R&D Systems Europe Ltd., Oxon, UK), according to the manufacturer's protocol. IFN-γ in restimulated cell culture supernatants was measured by ELISA using purified rat α-mouse IFN-γ as capture antibody and biotin-conjugated rat α-mouse IFN-γ as detection antibody followed by incubation with horseradish peroxidase-conjugated streptavidin (Zymed/Invitrogen, Taastrup, Denmark) and TMB Plus Ready-to-use substrate (Kem-En-Tec, Taastrup, Denmark). The reaction was stopped with 0·2 M H_2SO_4 and the absorbance was read at 450 nm.

Antibody detection

Detection of antibodies in the sera from immunized mice was performed using ELISA. In short, 96-well MaxiSorp plates were coated with 0·5 μg/ml of Ag85B–ESAT-6 in 15 mM Na_2CO_3, 35 mM $NaHCO_3$ (pH 9·7), overnight at 4°. The plates were blocked in PBS containing 2% (w/v) bovine serum albumin and sera were added in duplicate serial dilutions. Specific antibodies were detected following incubation with rabbit α-mouse IgG1 or IgG2a conjugated to horseradish peroxidase (Zymed/Invitrogen), and TMB Plus Ready-to-use was used as the substrate. The reaction was stopped with 0·2 M H_2SO_4 and the absorbance (optical density) was read at 450 nm.

Statistical analysis

Differences between the two adjuvant treatments were determined statistically using the *t*-test.

RNA isolation and microarray analysis

For the microarray analysis, PECs were stored at −20° in RNAlater (Qiagen) until required for isolation of RNA, which was performed using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. The quality and quantity of RNA was evaluated using the RNA 6000 Nano LabChip kit and the Agilent 2100 bioanalyzer (Agilent Technologies, Nærum, Denmark). Messenger RNA (mRNA) amplification, biotin-labelling and purification of amplified RNA (aRNA) was performed using the MessageAmp II aRNA kit (Ambion, Applied Biosystems, Nærum, Denmark). Equal amounts of aRNA were applied to GeneChip® Mouse Genome 430A 2.0 Arrays (Affymetrix, High Wycombe, UK) using the protocol supplied by the manufacturer.

Bioinformatics

Scanned Affymetrix gene chip CEL files were analyzed using the statistical language R (version 2.4.1) with the use of the Bioconductor packages 'affy', 'mouse430a2' and 'annotate' (http://www.bioconductor.org). Normalization was performed using the non-linear Qspline method,[28] expression indexes were calculated using the robust multi-array average method (RMA)[29] and statistical testing was performed using probe-level *t*-tests in an approach similar to that taken by Lemon and co-workers.[30] Volcano plots[31] based on all possible combinations were used to set the level of statistical significance, which was subsequently used to determine which genes were differentially expressed. Hypergeometric testing based on gene ontologies defined by the GO Consortium was used to determine which biological processes were most significantly over-represented in the list of differentially expressed genes.[32]

Results

$Al(OH)_3$ and DDA/MPL induce polarized immune responses

In the present study we investigated the innate signals preceding Th1/Th2 differentiation using the two model adjuvants $Al(OH)_3$ and DDA/MPL. These two adjuvants were evaluated *in vivo* to confirm their respective capacities to induce Th1 or Th2 antigen-specific responses using a standard subcutaneous immunization protocol. Therefore, the mycobacterial fusion protein Ag85B–ESAT-6 was adjuvanted in either DDA/MPL or $Al(OH)_3$ and injected three times, at 2-week intervals, into two different mouse strains. As expected, this gave rise to a polarized immune response with either a distinct Th1 (DDA/MPL) or Th2 [$Al(OH)_3$] profile: the formulation with DDA/MPL led to the release of high levels of antigen-specific IFN-γ, whereas high amounts of IL-5 were found only in the $Al(OH)_3$-vaccinated group (Fig. 1a). This difference in the overall immune profile was also reflected in the antibody subclass ratio where a high IgG2a : IgG1 ratio is commonly associated with Th1 responses and a lower ratio is associated with Th2 responses (Fig. 1b). The response to the two adjuvants was qualitatively very similar in BALB/c and C57BL/6 mice, with almost identical cytokine profiles and antibody responses observed.

Adjuvant-induced recruitment patterns

The adjuvant-induced innate signals preceding T-helper cell differentiation were subsequently investigated at the site of injection. As we had achieved similar Th1/Th2 profiles in BALB/c and C57BL/6 mice, we continued this more detailed analysis in the BALB/c strain only. We injected the adjuvants in the absence of antigen into the peritoneal cavities and isolated the cells recruited (the PECs). After 26 hr, we detected a major change in the FSC-SSC profiles of the two adjuvant groups compared with non-injected naïve mice (Fig. 2a). This was confirmed by differential staining, which showed increased

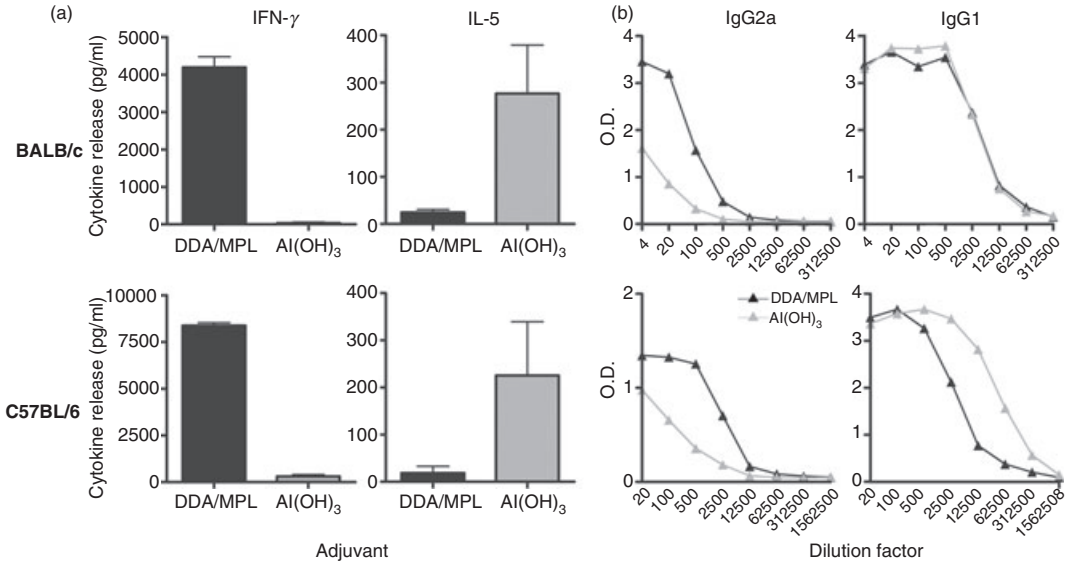

Figure 1. Dimethyldioctadecylammonium/monophosphoryl lipid-A (DDA/MPL) and aluminium hydroxide [$Al(OH)_3$] adjuvants induce antigen-specific T-helper 1 (Th1) and T-helper 2 (Th2) responses, respectively. BALB/c or C57BL/6 mice were immunized three times with Ag85B–ESAT-6 in either DDA/MPL (black bars/symbols) or $Al(OH)_3$ (grey bars/symbols). (a) One week after the final immunization the amount of interferon-γ (IFN-γ) and interleukin-5 (IL-5) in the supernatants of pooled blood lymphocytes from six mice restimulated with antigen was determined by enzyme-linked immunosorbent assay (ELISA). Bars represent the means of triplicate determinations and error bars represent the standard error of the means. (b) Four months after the final immunization, antigen-specific IgG2a and IgG1 antibody responses in pooled serum from four mice were determined by enzyme-linked immunosorbent assay (ELISA). The cytokine levels of naïve control mice were < 0·30 pg/ml and the antibody absorbance optical density (OD) levels were < 0·1. The results are representative of at least three individual experiments.

numbers of polymorphonuclear granulocytes, mainly neutrophils, after injection of both adjuvants (Fig. 2b) and approximately three times more cells in total. After $Al(OH)_3$ injection, a distinct population with an intermediate FSC (FSC^{int}) profile, but a high SSC (SSC^{hi}) profile, was found in the inflammatory exudate (Fig. 2a). This correlated with the differential staining, which demonstrated a small, but clearly detectable, population of eosinophils in the exudate from these mice (Fig. 2b). As distinction of murine eosinophils by flow cytometry can be problematic, we sorted the FSC^{int} SSC^{hi} cells, as well as the FSC^{int} SSC^{int} population as a control, by fluorescence-activated cell sorting (FACS) (Fig. 2c) and performed a differential staining, which confirmed distinct eosinophilic (SSC^{hi}) and neutrophilic (SSC^{int}) morphologies of the two populations (Fig. 2d). A specific marker for eosinophils is lacking in mice and they can therefore be difficult to distinguish from peritoneal differentiating monocytes and neutrophils if using Gr-1 (Ly-6G/C, a commonly used marker for polymorphonuclear granulocytes), because their FSC-SSC profiles overlap when they differentiate or become activated. However, the α2-integrin, CD49b, which is commonly used as a marker for murine NK cells, is also expressed on eosinophils (Fig. 2e). As NK cells and eosinphils are easily distinguished on their SSC characteristics, and CD49b is not found on neutrophils or monocytes, CD49b was used as a marker for murine eosinophils.

Cells and chemokines at the site of injection

The distinct FSC-SSC profiles observed after injection of the two adjuvants were further characterized quantitatively over time by surface-staining for markers of different cell types (Fig. 3a). Confirming the differential staining, the predominant cells were, shortly after injection of either adjuvant, neutrophilic granulocytes. Furthermore, the $Al(OH)_3$ group was characterized by the recruitment of high numbers of eosinophils at 26 hr after adjuvant injection. By contrast, DDA/MPL had recruited significantly more monocytes and mature macrophages at the same time-point. Another subset that was recruited preferentially by DDA/MPL was NK cells and at 26 hr their numbers were significantly higher than in the $Al(OH)_3$ group. In addition, the NK cells from DDA/MPL-injected mice were activated to a higher extent, as assessed by the degree of CD69 expression (Fig. 3c). Before adjuvant injection (time = 0 corresponds to naïve mice), B cells and DCs were common in the peritoneal cavity but their numbers rapidly declined after injection of the adjuvants. However, a small, but significant, increase of B cells and DCs 26 hr after injection of $Al(OH)_3$ was observed, although the levels were still lower than in naïve mice. In this antigen-free model, the number of T cells was also higher in naïve mice, in particular at the early time-points.

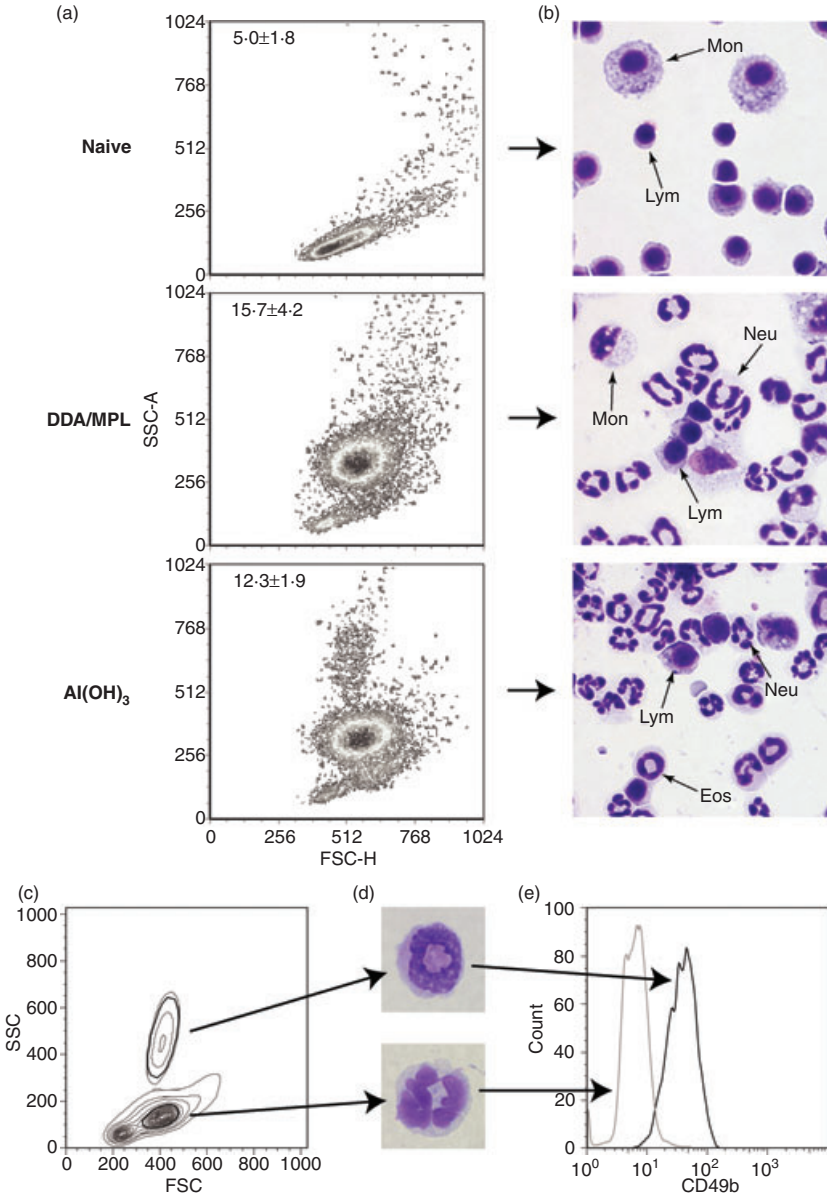

Figure 2. Adjuvant-induced inflammatory exudates. (a) Forward scatter (FSC)-side scatter (SSC) density plots of peritoneal exudate cells (PECs) from a naive mouse and from mice injected intraperitoneally (i.p.) with dimethyldioctadecylammonium/monophosphoryl lipid-A (DDA/MPL) or aluminium hydroxide [$Al(OH)_3$] 26 hr previously. The average number (± standard deviation) of PECs ($\times 10^6$) harvested from 3–10 mice is indicated in the top left corners of each plot. (b) As in (a) but PECs were stained with Hemacolor and analyzed by light microscopy for determination of monocytes/dendritic cells/macrophages (Mon), lymphocytes (Lym), neutrophils (Neu) and eosinophils (Eos). Images were recorded at 400 × magnification. (c) Contour plot of PECs from a mouse injected with $Al(OH)_3$ 26 hr previously. (d) The cells in (c) were sorted according to their FSC-SSC profile and stained with Hemacolor; one representative cell at 1000 × magnification is shown for each of the sorted populations. (e) The expression of CD49b on the two sorted populations, eosinophils (SSC^{hi}) and neutrophils (SSC^{int}), was determined by flow cytometry.

The peritoneal lavage was harvested in parallel to investigate the expression of selected chemokines and cytokines by CBA and ELISA. In general, DDA/MPL induced much higher quantities of the various cytokines and chemokines investigated and the only cytokine found in higher quantities in mice injected with $Al(OH)_3$ was IL-5 (Fig. 3b). With the exception of IL-5 and CCL24 (Eotaxin-2), which remained at or below the relatively high background level, the amounts of all cytokines and chemokines investigated in the peritoneal lavage were 2–50-fold higher after injection of DDA/MPL compared with $Al(OH)_3$ (Fig. 3b). The Th1/Th2-associated cytokines, IL-12p70 and IL-4, as well as IL-17, which is associated with inflammation and neutrophil homeostasis, were below the level of detection, whereas IFN-γ levels were repeatedly very low and only just detectable after injection with DDA/MPL (data not shown).

Gene expression profiles

We also investigated the expression of genes for cytokines, chemokines and chemokine receptors in a microarray analysis on mRNA extracted from the PECs of mice injected with the adjuvants 26 hr previously (Fig. 4). This time-point was chosen to ascertain a sufficient number of cells to achieve a high enough amount of mRNA for the subsequent microarray analysis to be carried out on indi-

vidual mice and thus strengthen the statistics of the experiment. The microarray data from the experiment have been submitted to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae). Both adjuvants induced the expression of several granulocyte and monocyte chemoattractants and activators (*Csf1*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*, *Ccl12* and *Cxcl7*) and also *Il1b* (Fig. 4a). However, in confirmation of its much stronger inflammatory potential, the injection of DDA/MPL induced the expression of genes for a range of C-X-C chemokines, including the NK- and T-cell-attracting C-X-C chemokine ligand (CXCL) 9 [monokine induced by IFN-γ (MIG)], CXCL10 [IFN-γ-inducible protein-10 (IP-10)], CXCL11 (IP-9), the C-C chemokine CCL5 [regulated on activation normal T-cell expressed and secreted (RANTES)], as well as the neutrophil-promoting granulocyte colony-stimulating factor (G-CSF) and several Th1-associated cytokines, including IFN-γ, IL-15 and TNF-α. In contrast to the large panel of genes expressed after injection of DDA/MPL, only a few genes were selectively expressed after injection of $Al(OH)_3$. Among these were the genes for CCL9 [macrophage inflammatory protein-1γ (MIP-1γ)] and CCL6 (C10). A hierarchical cluster analysis of the overall microarray results (~14 000 genes) illustrated that based on their entire gene-expression profiles, all nine individual mice clustered according to their treatment (Fig. 4b). In addition, this analysis demonstrated that the $Al(OH)_3$-induced gene-expression profile more closely resembled that of naïve mice than that of DDA/MPL-injected mice. Considering the entire microarray, DDA/MPL up-regulated more than twice as many genes as $Al(OH)_3$, and 75% of the genes up-regulated by $Al(OH)_3$ were also up-regulated by DDA/MPL (Fig. 4c). In addition, most of the genes significantly up-regulated after injection of DDA/MPL were more highly expressed in the DDA/MPL group compared with the $Al(OH)_3$ group (Fig. 4d). This was in contrast to the genes up-regulated by $Al(OH)_3$ (Fig. 4e).

The genes that were up-regulated or differentially expressed after injection of the adjuvants were subjected to a hypergeometrical test in which it was tested if the

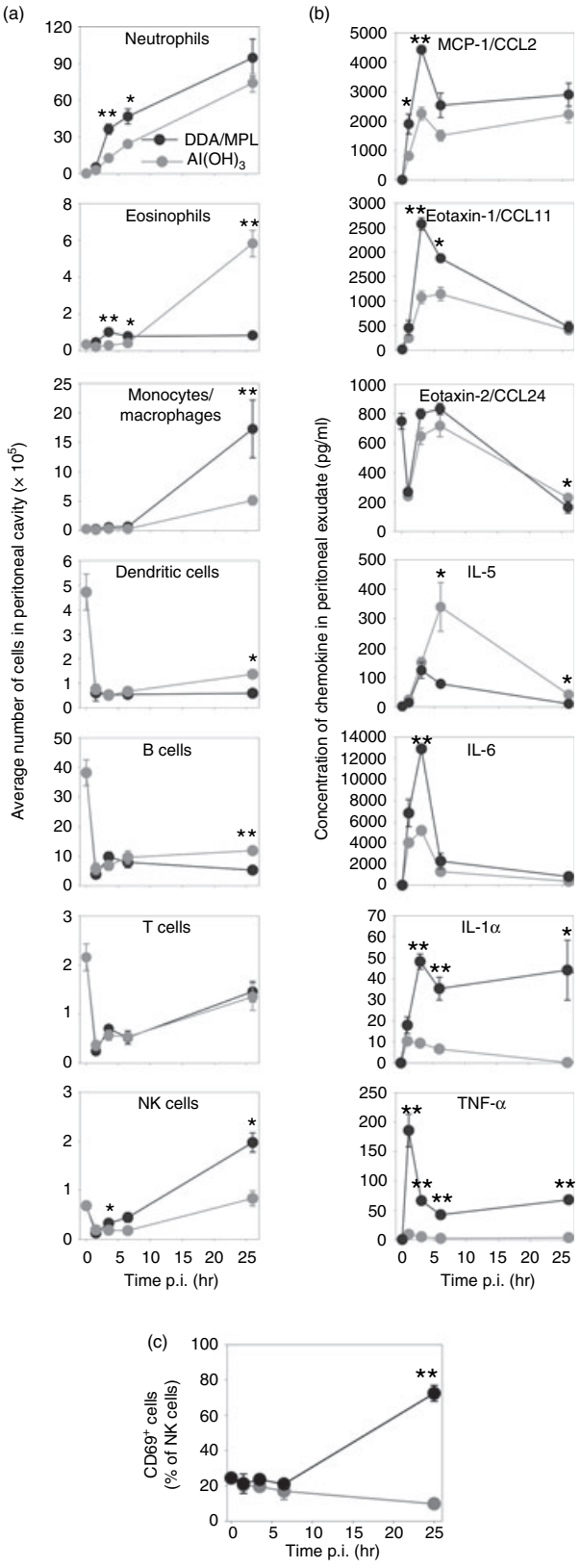

Figure 3. Cell types and cytokines/chemokines induced by T-helper 1 (Th1)- and T-helper 2 (Th2)-polarizing adjuvants. (a) Peritoneal exudate cells (PECs) were harvested at the indicated time-points after intraperitoneal (i.p.) injection of either dimethyldioctadecylammonium/monophosphoryl lipid-A (DDA/MPL) (black) or aluminium hydroxide [$Al(OH)_3$] (grey) and analyzed by flow cytometry. (b) Cytokines/chemokines expressed at the site of adjuvant injection. Expression of cytokines/chemokines in the peritoneal lavage fluid was investigated by cytometric bead array (CBA) or enzyme-linked immunosorbent assay (ELISA). CCL, C–C chemokine ligand; IL, interleukin; TNF-α, tumour necrosis factor-α. (c) The percentages of natural killer (NK) cells from (a) expressing CD69. Each data point represents the average of three individual mice and the error bars show the standard error of the mean. Statistically significant differences between the two groups have been indicated: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. The results are representative of three experiments. p.i., postinjection.

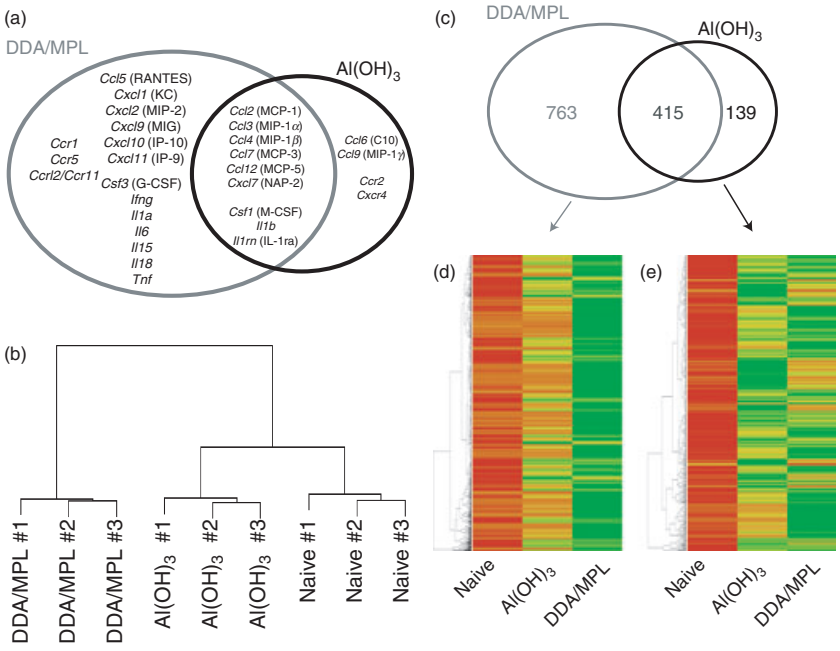

Figure 4. Hierarchical clustering and specifically up-regulated genes. Mice were injected intraperitoneally (i.p.) with dimethyldioctadecylammonium/monophosphoryl lipid-A (DDA/MPL) or aluminium hydroxide [$Al(OH)_3$], and the peritoneal exudate cells (PECs) were harvested 26 hr later and their gene-expression profiles were analyzed on Affymetrix microarrays. (a) Up-regulated genes for chemokines, chemokine receptors and cytokines after injection of the indicated adjuvants [grey circle: DDA/MPL; black circle: $Al(OH)_3$]. Genes up-regulated after injection of both adjuvants are shown in the overlapping part of the circles. Common names of the chemokines are shown in parenthesis. (b) Comparison of the expression profile of each microarray chip (each chip representing one individual mouse, corresponding to the nine branches), showed that the mice clustered according to their treatment. The cluster dendrogram is based on the Euclidian distance. (c) The number of up-regulated genes after injection of the indicated adjuvants [grey circle: DDA/MPL; black circle: $Al(OH)_3$]. The number of genes up-regulated after injection of both adjuvants is shown in the overlapping part of the circles. (d) The genes significantly up-regulated after injection of DDA/MPL are represented in a clustered heatmap based on the average gene-expression intensities of three individual mice per group. Red represents low expression, yellow represents intermediate expression and green represents high expression of the genes. (e) As in (d) but the heat map is based on the genes up-regulated after injection of $Al(OH)_3$. Up-regulated genes were determined at the level of statistical significance ($P < 0{\cdot}05$) as determined by Volcano plots.[31] KC, keratinocyte-derived chemokine; NAP, neutrophil-activating peptide.

genes belonging to known biological processes were over-represented compared with their distribution on the microarray (functional annotation). The five most statistically significant biological processes are listed in Table 1. We found that both adjuvants induced genes involved in immune and inflammatory responses, but DDA/MPL also induced genes involved in cytokine and chemokine signalling and apoptosis ('DDA/MPL > Naïve'), whereas $Al(OH)_3$ induced genes related to metabolism ['$Al(OH)_3$ > Naïve']. However, when the two adjuvant groups were compared in a separate test, it became clear that DDA/MPL induced more genes involved in immune and inflammatory responses than $Al(OH)_3$ ['DDA/MPL > $Al(OH)_3$'], whereas $Al(OH)_3$ induced more genes involved in energy transport and synthesis of proteins ['$Al(OH)_3$ > DDA/MPL'].

Discussion

The cytokine milieu that is responsible for the polarization of T cells into either a Th1 or a Th2 phenotype has been the subject of thorough *in vitro* studies and, although this distinction is less strict and more complex *in vivo*, the studies clearly demonstrate that the presence of IFN-γ and/or IL-12 during T-cell differentiation leads to a Th1 profile, whereas the presence of IL-4 leads to a Th2 profile.[33–35] More recently, the mechanisms behind the reciprocal regulation of Th1 and Th2 responses have been unravelled at the genetic level: activation of the signal transducer and activator of transcription (STAT)1 and STAT4 signalling pathways results in IFN-γ and IL-12 production and suppression of the STAT6 pathway, which would have led to IL-4 production and *vice versa*.[36–38] At the cellular level, several studies have attempted to clarify the activity of adjuvants by studying their effect on antigen-presenting cells (APCs) but this is an oversimplification that does not take into consideration the complexity of the intact organism where multiple inter-related mechanisms act in concert to induce, propagate and maintain the immune response.

In our study we wanted to investigate the initial effect of two polarizing adjuvants on the immune system *in vivo*.

Table 1. Functional annotation

Up-regulated processes		Differentially expressed processes	
DDA/MPL > Naïve	$Al(OH)_3$ > Naïve	DDA/MPL > $Al(OH)_3$	$Al(OH)_3$ > DDA/MPL
Immune response	Inflammatory response	Immune response	Protein biosynthesis
Inflammatory response	Glycolysis	Inflammatory response	Ribosome biogenesis
Cytokine and chemokine mediated signalling pathway	Pentose-phosphate shunt	Ag processing, endogenous Ag via MHCI	Tricarboxylic acid cycle
Actin cytoskeleton organization and biogenesis	Immune response	Ag presentation, endogenous Ag	Ag processing, exogenous Ag via MHCII
Regulation of apoptosis	ATP biosynthesis	Defense response	Ribosome biogenesis and assembly

A hypergeometric test based on the microarray data was used to determine which biological processes were up-regulated (test comparing adjuvant group with naïve) or differentially expressed (test comparing the two adjuvant groups) in peritoneal exudate cells (PECs) 26 hr after injection of adjuvants. The five most significantly changed processes are listed according to descending significance. The biological processes were defined according to the Gene Ontology (GO) Consortium.

Ag, antigen; $Al(OH)_3$, aluminium hydroxide; DDA/MPL, dimethyldioctadecylammonium/monophosphoryl lipid-A; MHCI, major histocompatibility complex class I; MHCII, major histocompatibility complex class II.

We found that DDA/MPL induced a complex inflammatory influx that was dominated by neutrophils, APCs and NK cells. This response was associated with high concentrations of pro-inflammatory and Th1-associated cytokines as well as with several monocyte and granulocyte chemoattractants detectable at both the protein and gene-expression level. An early signal for the strong inflammation induced by Th1 adjuvants has been suggested to be pro-inflammatory cytokines secreted by NK cells, which, when activated, are able to produce large amounts of IFN-γ and thus drive Th1 differentiation.[39] Here, we found that the Th1-inducing adjuvant, DDA/MPL, recruited high numbers of NK cells to the site of injection. Furthermore, approximately 80% of these cells were activated, as assessed by their expression of CD69, whereas < 10% of the NK cells were activated after injection of $Al(OH)_3$. The ability of DDA/MPL to recruit NK cells could be mediated by chemokines such as the C-X-C chemokine receptor 3 (CXCR3) ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (IP-9),[40] which were all significantly expressed after injection of this adjuvant. In addition to the recruitment of NK cells, DDA/MPL seems to be a general activator of the immune system, in particular of the residential APCs and of the mesothelial cells lining the peritoneal cavity, most probably mediated through TLR4.[41,42] Activation through TLR4 induces TNF-α,[41] CCL-2 (MCP-1) and CXCL2 (MIP-2) expression[42] which, as observed, leads to recruitment of inflammatory monocytes causing a positive feedback that further accelerates the recruitment and results in macrophage differentiation. In addition, inflammatory monocytes can differentiate into DCs *en route* to the draining lymphoid organs,[43] thus providing a source of APCs capable of initiating subsequent antigen-specific responses.

In comparison, injection of the Th2 adjuvant, $Al(OH)_3$, led to a more limited inflammatory response with a delayed cellular influx, restricted almost exclusively to neutrophils and eosinophils, and to a less pronounced release of pro-inflammatory cytokines and chemokines. In contrast to Th1 responses, the components that induce Th2 responses are few and the mechanisms less well understood. In general, Th2 responses are associated with allergic reactions and infections with extracellular parasites, such as helminths, as well as vaccines adjuvanted with aluminium salts. Understanding how Th2 responses are induced have therefore been investigated in detail using aluminium salts but despite these efforts some controversy still exists.[19] Adsorption of antigen to the various aluminium compounds has long been accepted as important for their ability to induce good immune responses but this has recently been disputed.[44,45] Similarly, the significance of a depot of antigen at the injection site is still unclear; as is the effect on APCs.[21,22,24,25] In a recent study, aluminium, in the form of Imject® Alum, was shown to induce uric acid release at the injection site, leading to recruitment of inflammatory monocytes.[15] Similarly, we observed increased numbers of monocytes and macrophages after injection of $Al(OH)_3$ but we also found that the numbers were much lower than after injection of DDA/MPL and we did not observe increased numbers of mature DCs. The differences may be explained by the use of different sources of aluminium, because we used the licensed Alhydrogel, which is different from Imject® Alum that, besides aluminium, contains equal amounts of magnesium hydroxide.[46] *In vitro*, TLR ligands are required for aluminium compounds to induce IL-1β production by macrophages.[17,18] Nevertheless, like Mosca *et al.*,[47] we found that $Al(OH)_3$ induced the expression of *Il1b* at the site of injection. This lack of dependence on TLR ligands *in vivo* is probably an effect of the inevitable local necrosis caused

by injection of aluminium compounds and the subsequent release of uric acid and activation of the inflammasome.[15,48]

Characteristically, $Al(OH)_3$ induced the accumulation of not only neutrophils but also of eosinophils at the injection site. This is in agreement with the work of Walls[49] and with studies of aluminium-induced experimental allergy.[50] Furthermore, IL-5 was the only cytokine or chemokine found to be selectively expressed early after injection of $Al(OH)_3$. IL-5 is strongly involved in eosinophilopoiesis and has a known synergistic effect on the ability of eotaxins to attract eosinophils.[51,52] That the observed eotaxin levels were lower after injection of $Al(OH)_3$ than of DDA/MPL highlights the importance of IL-5 for optimal recruitment of eosinophils. That eosinophils may play a role in the ability of $Al(OH)_3$ to induce optimal Th2 responses is suggested in a study where aluminium-induced B-cell responses *in vivo* required not only IL-4 but also the presence of eosinophils and/or an unidentified myeloid Gr-1^+ cell population.[53]

Based on the microarray gene-expression profiles and the cluster analysis, we found that injection of $Al(OH)_3$ resulted in an immunological profile which was more closely related to that of naïve mice than to that of DDA/MPL-injected mice. That Th2 responses are induced under more 'immunologically silent' conditions compared with Th1 responses is consistent with a default Th2 pathway, as suggested several years ago by Jankovic *et al.*,[54] which states that during infectious disease a Th2 response will develop in the absence of Th1-promoting factors. More recently, the same group has found that parasite-induced Th2 responses are related to a dampening of Th1-stimulating immune reactions and a lack of MyD88-signalling.[55] In addition, Sun *et al.*[56,57] have found that Th2 responses arise when Th1 or TLR signals are lacking and, although it has been demonstrated that if IL-4 is blocked following immunization with aluminium a Th1-like response arises,[58] Th2 responses can still be found in the absence of IL-4 or IL-4 signalling *in vivo*.[26,27] Our microarray experiment, which was based on individual mice, indicates that the initial immune response to $Al(OH)_3$ is more delayed compared with that of DDA/MPL because metabolic processes (e.g. glycolysis, ATP biosynthesis, protein biosynthesis, tricarboxylic acid cycle) precede the energy-consuming and protein-dependent immunological processes (e.g. immune response, inflammatory response, cytokine/chemokine signalling). As *de novo* synthesis of MHC class I is delayed compared with MHC class II in DCs infected with bacteria,[59] it seems likely that the cells from DDA/MPL-injected mice were more advanced because they had a higher expression of genes related to endogenous antigen processing.

Our findings emphasize that adjuvants affect not just APCs but also the other cells at the injection site, leading to a rapid selective cellular recruitment. Early after injection, the Th2 adjuvant, $Al(OH)_3$, selectively induced the release of IL-5 and a subsequent influx of eosinophils. Otherwise, $Al(OH)_3$ induced a limited inflammatory response compared with the Th1 adjuvant, DDA/MPL, which was highly active, inducing a range of pro-inflammatory cytokines and chemokines and the consistent influx of inflammatory monocytes, macrophages and activated NK cells. Interestingly, at these early time-points, and in the absence of antigen, we were unable to detect the classical Th1- and Th2-associated cytokines IL-12 and IL-4 at the injection site, suggesting that these cytokines are expressed either later or, when antigen is present, in the draining lymphoid organs as part of the antigen-specific response. Understanding the precise molecular mechanisms that initiate these complex cascades and result in an immune response with the desired profile will allow the development of highly defined, tailored adjuvants that in the future can form the basis of new, successful vaccines.

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Disclosures

None.

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