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AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity

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Adjuvant System 04 (AS04) combines the TLR4 agonist MPL (3-O-desacyl-4′-monophosphoryl lipid A) and aluminum salt. It is a new generation TLR-based adjuvant licensed for use in human vaccines. One of these vaccines, the human papillomavirus (HPV) vaccine Cervarix, is used in this study to elucidate the mechanism of action of AS04 in human cells and in mice. The adjuvant activity of AS04 was found to be strictly dependent on AS04 and the HPV Ags being injected at the same i.m. site within 24 h of each other. During this period, AS04 transiently induced local NF-κB activity and cytokine production. This led to an increased number of activated Ag-loaded dendritic cells and monocytes in the lymph node draining the injection site, which further increased the activation of Ag-specific T cells. AS04 was also found to directly stimulate those APCs in vitro but not directly stimulate CD4 dendritic cells and monocytes in the lymph node draining the injection site, which further increased the activation of Ag-specific T cells.


The development of new vaccines has highlighted the need for new strategies to enhance and tailor the immune response for effective and long-lasting protection. In particular, vaccines based on soluble recombinant Ags typically require adjuvants to enhance an Ag-specific adaptive immune response, i.e., a T cell and Ab response (1). The induction of an optimal innate immune response is also associated with an enhanced adaptive immune response. In light of the recent advances in understanding the receptors and associated agonists responsible for the induction of the innate immune response (e.g., TLRs), a new generation of adjuvants incorporating these agonists has been engineered for new prophylactic and therapeutic vaccines (2). Understanding how adjuvants work at the molecular level and more importantly in vivo is critical for the development of safe and more efficient vaccines.

Adjuvant System 04 (AS04)6 consisting of MPL (3-O-desacyl-4′-monophosphoryl lipid A) adsorbed onto a particulate form of aluminum salt is one of these new generation adjuvants now licensed for use in humans (1). AS04 is currently a component in two licensed vaccines, one against the cervical precancerous lesions and cancer containing virus-like particles (VLPs) of the L1 protein from human papillomavirus (HPV)-16 and HPV-18 oncogenic strains of HPV (Cervarix) (3–5) and the second against hepatitis B virus (Fendrix) (6). A third vaccine against herpes simplex 2 virus is in phase III clinical trials.

MPL is a detoxified derivative of the LPS isolated from the Gram-negative bacterium Salmonella minnesota R595 strain (7–9). LPS has been found to function as a specific agonist of TLR4 (10, 11). MPL signals via TLR4 (12–14), although one report describes signaling via TLR2 (13). TLR4 stimulation can contribute to the activation of the innate immune response, by activating NF-κB transcriptional activity and the subsequent expression of proinflammatory cytokines, such as TNF-α and IL-6 (15). These cytokines can in turn enhance the adaptive immune response by stimulating the maturation of APCs while repressing the tolerance response through the inhibition of regulatory T cell activity (16). MPL is generally reported to promote IFN-γ production by Ag-specific CD4+ T cells, therefore skewing the immune response toward a Th1 profile (9). A Th1 immune response is required for effective protection against intracellular pathogens.

Aluminum salt has a long history of being used as an adjuvant (17) and has been shown as having a bias toward promoting Abs and a Th2 response. This type of immune response is effective against extracellular pathogens, but not against intracellular pathogens. Due to its particulate nature, aluminum salt is considered to act as a depot for vaccine Ag components, which enhances Ag

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FluO-OVA, Alexa Fluor 647-labeled OVA; HEK, human embryonic kidney; CBA, cytokine bead array.

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6 Abbreviations used in this paper: AS04, Adjuvant System 04; VLP, virus-like particle; HPV, human papillomavirus; DC, dendritic cell; BMDC, bone marrow DC.
uptake by APCs. At a molecular level, aluminum salt has been found to stimulate Nlrp3, a component of the inflammasome (18–20). The inflammasome functions as an intracellular multiprotein platform for the recruitment and activation of caspase-1 and the subsequent processing of proform of cytokines such as IL-1β or IL-18. In vitro, this requires the pretreatment of APCs with TLR ligands, such as MPL, as aluminum salt alone is reported not to be able to induce the transcription of IL-1β or IL-18 genes. There is therefore a theoretical basis to suggest that MPL and aluminum salt synergize in AS04 to produce elevated levels of IL-1β or IL-18. However, whether Nlrp3 and IL-1β/IL-18 play a role in the adjuvant activity of aluminum salt alone is a matter of debate (21).

In the Cervarix vaccine, AS04 is formulated with the hydroxide salt of aluminum. The Ag component of the vaccine is made up of VLP incorporating the major coat protein L1 from HPV-16 and HPV-18 strains (1). Recent work has shown that AS04, compared with an adjuvant containing only aluminum salt, induced a higher and long-lasting immune response to identical VLP Ag components of the respective HPV vaccines (22). Both AS04 and MPL, but not aluminum salt alone, were found to induce TNF-α secretion in monocytes suggesting that these in vivo differences in immunogenicity arose from the capacity of MPL to stimulate TLR4 signaling. Therefore the objective of this study was to assess the contribution of MPL in AS04 response and whether the MPL-specific response was modulated by its adsorption on aluminum salt in the context of the Cervarix vaccine. To achieve this, the response induced by the vaccine and its separate components were investigated in vitro on human cells and in vivo in mice following i.m. injection. In these experiments, the spatial and temporal parameters for AS04 to function as an efficient adjuvant were defined and related to how the vaccine and its components stimulated TLR4 signaling, cytokine expression, and APC activation.

Materials and Methods

Vaccine formulation

A 50-μl dose of vaccine contained 5 μg of clinical grade malt (purified from S. minnesota), 2 μg each of HPV-16 and HPV-18 L1 VLPs (Ag for preparation of the Ags, see Ref. 22) and 50 μg of aluminum hydroxide (Brenntag). The 50-μl vaccine dose used in this study represents one-tenth of the routine Cervarix vaccination dose in human subjects. In some experiments, 5 μg of OVA (Calbiochem) or Alexa Fluor 647-labeled OVA were added to the vaccine formulation to compare the effect of ALG and MPL. In all experiments, antigenicity was determined by ELISA, following the protocol previously described (22), in serum samples from individual blood donor donations. Mononuclear cells were obtained by centrifugation over Ficol-Hyphaque gradients. PBMCs were cultured in complete medium containing RPMI 1640 supplemented with 50 μM 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1% nonessential amino acids (Life Technologies), and 5% FCS (PAA Laboratories). To measure intracellular cytokine production in monocytes, PBMCs at indicated concentrations were stimulated in triplicate in 96-well plates at 37°C for 30 min before adding brefeldin A for an additional 5.5 h. Cells were then washed and detached using 2 mM EDTA in PBS. After surface staining with CD14 Abs, cells were labeled intracellularly for IL-6 and TNF-α and analyzed by flow cytometry. In some experiments, PBMCs were preincubated for 1 h at 37°C with 2 μg/ml blocking anti-TLR4 Ab (clone H1A125; ebioscience), an anti-TLR2 Ab (clone TL2.1; ebioscience), or both. Peptidoglycan from Staphylococcus aureus (FLUKA; Sigma-Aldrich) was used as TLR2 agonist. For the evaluation of IL-1β production, 1 × 10^6 PBMCs were stimulated in 96-well plates for 18 h and supernatants were collected in triplicates. The caspase-1 inhibitor Y-VAD (Calbiochem) was used at 10 μg/ml. Human IL-1β was measured by cytokine bead array (BD Biosciences). TLR-stimulated expression of secreted alkaline phosphatase. Transfected HEK 293 cells (InvivoGen) with the expression vectors encoding 1) TLR4, MD-2, and CD14, 2) TLR1 and TLR2, or 3) TLR2 and TLR6 were further stably transfected with the NF-κB reporter vector pNifty-2 secreted alkaline phosphatase (Invivogen). Cells were cloned by selection on the basis of appropriate elements. In vivo imaging, mice were killed by cervical dislocation and the dissected organs were homogenized in reporter lysis buffer (Promega) and cleared by centrifugation. Luciferase activity was quantified (conventional luminometry) in the supernatant by adding luciferin, ATP, and D-luciferin (Biosynth). Time from D-luciferin injection to imaging of NF-κB luciferase activity was performed noninvasively or in mice with surgically exposed lymph nodes following i.p. injection of luciferin (120 mg/kg; Biosynth). Time from luciferin injection to image acquisition was 10 and 25 min, respectively. Acquisition time was typically 1–2 min using the IVIS100 Imaging System (Xenogen), composed of a light-sealed sample chamber fitted with a heating platform (37°C) and cooled CCD camera (~10°C). Image acquisition and analysis were done with the accompanying IVIS100 software. During the imaging period, mice were anesthetized with 2.5% isoflurane. Immediately after in vivo imaging, mice were killed by cervical dislocation and the dissected spleen specimens were snap-frozen in liquid nitrogen. Individual frozen organs were homogenized in reporter lysis buffer (Promega) and cleared by centrifugation. Luciferase activity was quantified (conventional luminometry; Turner Instruments) in the supernatant by adding luciferin, ATP, and magnesium, following the manufacturer’s instructions (Promega). Luciferase activity was normalized to protein content (Bio-Rad).

Measurement of cytokines in mice

Cytokines were measured in murine muscle and serum samples. For each pooled muscle sample, injected gastrocnemius muscles were pooled and homogenized using an Ultraturrax (VWR) in 3 ml of PBS plus anti-protease inhibitor mixture (Sigma-Aldrich). The homogenates were then cleared by centrifugation at 14,000 rpm for 10 min, and supernatants were stored at −70°C before analysis. Serum samples were measured from individual
mice. Cytokine levels were measured according to the manufacturer’s recommendations by flow cytometry using CBA for IL-6, IL-12, TNF-α, IL-1β, CCL2, and CCL3 or by Meso Scale Discovery kit for CXCL1, IL-10, IL-12, or IFN-γ. IFN-α was measured by ELISA (PBL Biomedicals).

Preparation of mouse cells

Cell suspensions from the spleen and from the left and right axillary non-draining lymph nodes from each individual mouse and from pooled iliac lymph nodes were prepared using a Potter Homogenizer in medium (RPMI 1640, 10% FCS, 100 U/ml of penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-ME, 1 mM sodium pyruvate) and passed through a 100-μm filter. Cells were then washed and counted using a Multisizer (Beckman Coulter).

Measurement of OVA uptake

Bone marrow DCs (BMDCs) were prepared from bone marrow cells treated with mouse GM-CSF (10 ng/ml; R&D Systems) for 6 days. BMDCs were resuspended in complete medium at 1 × 10⁶ cells/ml and incubated in 96-well plates in triplicate with Fluoro-OVA-containing formulations at the indicated concentrations. Five hours later, the cells were washed extensively and the level of Fluoro-OVA uptake was analyzed by flow cytometry. In vivo, mice (n = 4; 4 pools of 6 mice/group) were immunized i.m. with Fluoro-OVA-containing formulations as indicated. After 24 h, cells were prepared from pooled iliac lymph nodes and analyzed by flow cytometry.

Measurement of Ag presentation to OVA-specific T cells

OVA-specific CD₄⁺ T cells were purified from pooled spleen and lymph nodes of OVA-TCR transgenic DO11.10 and OT-II mice using a CD4 T cell negative selection kit (Dynal Biotech) according to the manufacturer’s recommendations. Purified CD₄⁺ T cells were then stained with CFSE. Briefly, 1 × 10⁶ T cells/ml were incubated with 5 μM CFSE in RPMI 1640 for 10 min at 37°C. Cold RPMI 1640 plus 5% FCS was added to the cells to stop CFSE labeling and the cells were washed extensively.

BMDCs from BALB/c mice (for DO11.10 T cells) and C57BL/6 mice (for OT-II T cells) at 1 × 10⁵ cells/ml were incubated in complete medium with OVA-containing formulations at the indicated concentrations for 6 h in 24-well plates and washed extensively. Lymph node CD11c⁺ DCs were purified using CD11c⁺ selection kit (Miltenyi Biotec) from pooled iliac lymph nodes from mice that were immunized 1 day before with OVA-containing formulations (n = 24 mice per vaccination). Treated BMDCs or purified lymph node DCs were incubated with 1 × 10⁵ CFSE-labeled OVA-specific T cells in 96-well plates at the indicated DC to T cell ratio. After 3 days, the cells were washed and the proliferation of the T cells was assessed by flow cytometry.

Flow cytometry

Cells were resuspended in PBS, 1% FCS, and 1 mM EDTA and stained with the Abs described. To measure activation of human DCs, cells were stained using anti-CD86-FITC, anti-CD1a-PE, anti-CD83-allophycocyanin, and anti-CD14-PE. To measure activation of human immune cells, cells were first treated with 2.4G2 Ab for 5 min to block the Fc receptor and stained with the following Abs: anti-CD3 PE, anti-CD14-PE, anti-CD69-allophycocyanin, anti-MHC class II-PE, anti-CD40-PerCP, and anti-CD163-PE. To analyze T cell proliferation by CFSE dilution, the cells were stained with anti-CD4 allophycocyanin and the clonotypic anti-OVA TCR Ab KJ1.26 PE (DO11.10) and the combination of anti-TCR Vβ5 PE and Vα6 biotin (OT-II). Biotinylated Abs were revealed using PerCP-labeled streptavidin. All Abs were obtained from BD Biosciences. Fluorescent events were acquired using an LSR2 and analyzed using FACSDiva software (BD Biosciences).

Splenocyte stimulation by HPV-16 or HPV-18 L1 VLP Ags

Three groups of BALB/c mice (n = 12) were immunized i.m. (days 0 and 21) with 2 μg of HPV 16/18 L1 VLPs alone, or formulated with aluminum hydroxide (50 μg) or with AS04 (50 μg of aluminum hydroxide plus 5 μg of aluminum hydroxide (Alum), or both at indicated concentrations (μg/ml). PBS or positive controls (+ controls) in LPS (0.1 μg/ml) (top), PAM3CSK4 (5 μg/ml) (middle), and PAM2CSK4 (5 μg/ml) (bottom). Cells were plated at 2 × 10⁶ cells/well in 24-well plates 24 h before stimulation. Data represent the mean relative levels of secreted alkaline phosphatase activity as a measure of NF-κB activity (in triplicate cultures). Error bars describe SDs.

FIGURE 1. MPL acts as a TLR4 agonist. A, HEK cells transfected with tlr4, md2, and cd14 with tlr1 and tlr2; or with tlr2 and tlr6 were stimulated with MPL, aluminum hydroxide (Alum), or both at indicated concentrations (+ controls) in LPS (0.1 μg/ml) (top), PAM3CSK4 (5 μg/ml) (middle), and PAM2CSK4 (5 μg/ml) (bottom). Cells were plated at 2 × 10⁶ cells/well in 24-well plates 24 h before stimulation. Data represent the mean relative levels of secreted alkaline phosphatase activity as a measure of NF-κB activity (in triplicate cultures). Error bars describe SDs. B, The proportion of TNF-α- and IL-6-positive human CD14⁺ monocytes in PBMC cultures (5 × 10⁵ cells/well; 96-well plate) stimulated for 6 h with MPL (0.1 μg/ml), or peptidoglycan (PGN, 0.1 μg/ml), or in the presence of medium (control), an anti-TLR2- or an anti-TLR4-blocking Abs. Data represent geometric mean and the symbols represent the data points from each of the donor samples tested (n = 5 donors).
Two weeks after the second immunization, spleen cell suspensions were prepared from pooled mice (n = 4, 4 pools of 3 spleen samples/group). Cells were cultured at a final concentration of 5 x 10^6 cells/ml in 1 ml per flat-bottom 24-well plates with 1 g/ml L1 HPV-16 or HPV-18 Ag. Supernatants were harvested at 48 h later and tested for the presence of IFN-γ, IL-2, and IL-5 using a mouse CBA kit.

**Statistics**

Statistical analyses were performed on logarithmic transformed data. The Shapiro-Wilk test was used to confirm normality. The ANOVA and the Tukey’s test were applied to identify differences between treatment groups, except in the analysis of TLR responses in PBMCs and NF-kB activity for which the two-sided Dunnett’s test was applied. Statistical significance was assigned at the value for p ≤ 0.05. For the analysis of Ab titers, statistical significance was assigned at a value for p ≤ 0.05 and the difference between the groups was also >2-fold.

**Results**

**MPL acts as a specific TLR4 agonist**

LPS from the Gram-negative bacterium S. minnesota has been reported to be a specific TLR4 agonist (10, 11). MPL, a detoxified derivative of LPS, has also been shown to act through TLR4 (12), although one study reported some TLR2 activity (13). The contribution of TLR4 and TLR2 on MPL activity was therefore re-examined using clinical grade MPL alone or within AS04. This examination was done using HEK cells transfected with plasmids encoding TLR4 and its coreceptors MD2 and CD14, or encoding TLR2 and TLR1, or encoding TLR2 and TLR6 (Fig. 1A). Both MPL and AS04, but not aluminum hydroxide alone, induced NF-κB activity in the tlr4-md2-cd14 transfectants, confirming that MPL signals via TLR4.

**FIGURE 2.** AS04 stimulated human APCs but not T cells. A, The proportion of TNF-α-positive (open bars and symbols) and IL-6-positive (shaded bars and symbols) CD14+ monocytes in human PBMCs (n = 4 donors). PBMCs in 5 x 10^5 cells/well (96-well plate) were stimulated as in Fig. 1B with medium or a dose range of MPL or aluminum hydroxide at the indicated concentrations (in µg/ml). B, The level of IL-1β detected in culture supernatants of human PBMCs. PBMCs were stimulated with a dose range of MPL or aluminum hydroxide at the indicated concentrations (in µg/ml) for 18 h without and with caspase-1 inhibitor Y-VAd. Data represent geometric mean and the symbols represent the data points from each donor sample tested. C and D, Human monocyte-derived DCs in 2 x 10^5 cells/well (96-well plate) were stimulated with the full vaccine (HPV-16 and HPV-18 L1 VLPs/AS04), Ags (HPV-16 or HPV-18 L1 VLP/aluminum hydroxide) or MPL at the concentrations indicated (in µg/ml) (n = 2 or n = 4 mice). C, The levels of TNF-α and IL-6 after 18 h in the supernatant are shown. D, The proportion of CD1a+ DCs expressing the costimulatory molecules CD83 (open symbols) and CD86 measured by flow cytometry after 18 h. Data represent geometric means and the symbols represent the data points from each of donor samples tested. E, Human CD4+ T cells for n = 6 donors in 2 x 10^5 cells/well (96-well plate) were stimulated for 24 h with medium alone, MPL (10 µg/ml), anti-CD3 (1 µg/ml), or MPL (10 µg/ml), and anti-CD3 (1 µg/ml) and the proportions of CD69, IFN-γ, and CD40L-expressing cells relative to the anti-CD3 condition is represented as a box plot. The median of the n = 6 donors is shown (horizontal line), and results are described by the 1st and 3rd quartiles with the lowest and highest values shown by the whiskers.
NF-κB activity were detected in both tlr1/2 and tlr2/6 transfected cells.

To evaluate TLR4 and TLR2 responses in a more relevant model, PBMCs were stimulated for 6 h, and intracellular cytokines were measured in activated CD14⁺ monocytes. MPL induced the production of IL-6 and TNF-α by monocytes and this response was reduced when a TLR4 blocking Ab was added before MPL (p < 0.001, Fig. 1B). In contrast, an anti-TLR2 Ab had no effect on MPL-induced monocyte activation (p = 0.9514), whereas it inhibited monocyte activation by a TLR2 agonist, peptidoglycan (p < 0.01). Altogether, these results indicated that MPL either alone or adsorbed on aluminum hydroxide (as formulated in AS04) acts as a TLR4 agonist that is in agreement with what was reported by Tiberio et al. (14).

The stimulation of PBMCs with a dose range of AS04 or MPL similarly induced CD14⁺ monocytes to express IL-6 and TNF-α (Fig. 2A) compared with stimulation with aluminum hydroxide (p < 0.0001 and p < 0.001, respectively) in which IL-6⁺ and TNF-α⁺ monocytes remained at background levels. Therefore aluminum hydroxide in the AS04 preparation did not interfere with or appear to enhance the TLR4-specific proinflammatory cytokine production induced by MPL on human monocytes. However, there is a potential for MPL and aluminum hydroxide to synergize in the secretion of caspase-dependent cytokines, given their respective influences on transcription and maturation of these cytokines. As expected, the stimulation of PBMCs with a dose range of AS04 but not with aluminum hydroxide alone induced the secretion of IL-1β (p < 0.001), which was inhibited by the addition of the caspase-1 specific inhibitor Y-VAd (Fig. 2B). A similar induction of IL-1β was observed with MPL stimulation compared with aluminum hydroxide (p < 0.01). This indicated that MPL was sufficient to induce the caspase-1 dependent production of mature IL-1β. Therefore, the combination of MPL and aluminum hydroxide did not significantly synergize in the induction of cytokine secretion.

**MPL and AS04 stimulate human DCs but not human T cells**

DCs as well as T cells have been shown to express TLR4 (24, 25). Human DC stimulation with a dose range of MPL or the vaccine component both significantly induced the production of TNF-α and IL-6 and increased the surface expression of the costimulatory molecules CD83 and CD86, compared with HPV-16 or HPV-18 L1 VLPs adsorbed on aluminum hydroxide (Fig. 2D). Typically these responses were proportional to the concentration of MPL used. Therefore MPL retained its ability to activate DCs when formulated in the vaccine. Furthermore, MPL was the only component in the vaccine that drove this response as the HPV Ags adsorbed on aluminum hydroxide had no direct effect on these parameters of DC maturation.

Purified human CD4⁺ T cells were stimulated with MPL in the presence or absence of TCR engagement via anti-CD3 Abs (Fig. 2E). Consistent with the study by Ismaili et al. (25), CD40L was slightly increased in T cells but only upon concomitant TCR engagement. However, this difference was not found to be significant when tested in six donors. In addition, MPL did not significantly alter the expression of two other markers of T cell activation, i.e., CD69 and IFN-γ, without consideration of anti-CD3 stimulation. Therefore MPL was able to directly stimulate human monocytes and DCs, but unable to significantly stimulate T cells.

**Spatial and temporal colocalization of AS04 and Ags is required for an optimal immune response**

The superior adjuvant activity of AS04 over aluminum salt alone has been previously demonstrated in mice following vaccination with HPV-16/18 L1 VLPs (22). To compare the adjuvant activity of AS04 to MPL, C57BL/6 mice were injected with HPV-16/18 L1 VLPs adjuvanted with AS04 or MPL alone, twice at day 0 and day 14, and the humoral responses were analyzed 14 days later. The addition of aluminum hydroxide to MPL significantly enhanced the Ab response for both HPV-16 and HPV-18 L1 VLPs (from 7893 to 21614 EU/ml for HPV-16 and from 5758 to 25745 EU/ml for HPV-18; n = 20, p < 0.001), further demonstrating the added value of MPL and aluminum salt combination in the vaccine.

To identify the spatial and temporal requirements of AS04 adjuvant activity, different i.m. immunization protocols were followed in C57BL/6 mice (Fig. 3). Effectively the vaccine dose was split such that MPL per one-half dose of aluminum hydroxide was injected first and the Ags (HPV-16/18 L1 VLPs per one-half dose of aluminum hydroxide) were then injected 1 or 24 h later either into the same limb site or into a separate site on the contralateral limb. HPV-16/18 L1 VLPs, adjuvanted in AS04 (the whole vaccine) or adjuvanted with only an equivalent amount of aluminum hydroxide, were used as positive and negative controls. The production of anti-CD3 stimulation. Therefore MPL was able to directly stimulate human monocytes and DCs, but unable to significantly stimulate T cells.

**FIGURE 3.** AS04 induced high geometric mean titers (GMTs) compared with aluminum hydroxide alone, when the Ags were injected in the same site and within 24 h after the MPL/aluminum hydroxide injection. Anti-HPV-16 L1 VLP geometric mean titers (A) and anti-HPV18 L1 VLP geometric mean titers (B) were measured 14 days after i.m. immunization in C57BL/6 mice. The quantity of the vaccine component (MPL: aluminum hydroxide, and HPV-16 L1 VLP/HPV-18 L1 VLP) used in each immunization (first/second injection) was tabulated directly below the respective results. Error bars represent ± 95% confidence intervals (n = 18). *p < 0.0001 for significant differences by Tukey’s test between the respective geometric mean titer means and the value observed after the injection of the Ags adjuvanted with AS04 (VLPs/AS04).
negative controls respectively. The impact of the adjuvant activity on the humoral responses was assessed at 14 days after the first injection.

The Ab response was not significantly different when the Ags were injected 1 h after MPL and aluminum hydroxide at the same site compared with the whole vaccine. However, when the Ags were injected 24 h later than MPL and aluminum hydroxide, the Ab response was significantly lower (≤2.4-fold) than the response observed with the vaccine. When the Ags were injected after MPL and aluminum hydroxide but in the contralateral muscle, MPL and aluminum hydroxide did not have any impact on the immunogenicity of the Ags and the Ab titers were not significantly above the negative control (Ags adjuvanted with aluminum hydroxide alone). Therefore the superior adjuvant activity of AS04 vs aluminum hydroxide was dependent on the Ags being in the same location as AS04 within a period of 24 h.

The i.m. injection of MPL or AS04 leads to local NF-κB activation

A first evaluation of the vaccine localization in situ by immunohistochemistry indicated that MPL and the other vaccine components were found at the site of injection for at least 24 h following the vaccine injection (data not shown). Only a limited quantity of Ag and no MPL could be detected in the lymph nodes, suggesting that the vaccine components remain primarily local to the site of injection.

Because MPL has been shown to induce NF-κB activation (13, 25), NF-κB activity was used as a readout for the functional response to MPL to further characterize the localization of MPL. This response was measured using a transgenic mouse model that reveals NF-κB activity by expression of a luciferase reporter gene (23).

The injection of AS04 into the right gastrocnemius muscle was compared with the injection of PBS into the left gastrocnemius muscle (Fig. 4A). AS04 resulted in higher NF-κB activity (p < 0.01) in right hind limb compared with the contralateral limb at 5 h, but not at 22 h postinjection (Fig. 4A). This indicated that AS04 transiently stimulated TLR4 signaling at the injection site.

Therefore, to examine the direct responses to MPL, NF-κB activity was also measured in surgically exposed lymph nodes at 5 h postinjection. Compared with PBS, the injection of MPL and AS04 resulted in a 4.7-fold (p < 0.001) and 2.8-fold (p < 0.05) higher NF-κB activity, respectively, in the hind leg draining iliac lymph node (Fig. 4B). In the right inguinal lymph node, the injection of MPL also resulted in 4.0-fold higher (p < 0.05) NF-κB activity (Fig. 4B), in contrast to the left inguinal lymph node that does not drain the injection site.

As a measure of systemic responses to MPL, NF-κB activity was examined in spleen homogenates (Fig. 4C). MPL i.v. injection, but not MPL or AS04 i.m. injection, resulted in NF-κB activity in the spleen (p < 0.001). Therefore, following AS04 i.m. injection, the direct response to MPL was restricted to the injection site and draining lymph node.

Local cytokine induction by MPL and AS04

NF-κB activity is associated with an innate immune response that results in the induction of cytokines. Proinflammatory cytokines, secreted by resident and recruited cells, directly stimulate cells that then present the Ag in the draining lymph node. Chemokines play a key role in inducing the recruitment of various immune cells at the injection site. The contribution of MPL and the other vaccine components to the innate immune response at the injection site was therefore further examined by measuring the levels of proinflammatory cytokines (IL-6, TNF-α, and IFN-α) and chemokines (CCL2/MCP-1 and CCL3/MIP-1α) in homogenates prepared from injected muscle at 3, 6, and 24 h and 7 days postinjection (Fig. 5A). PBS was injected as a baseline negative control. However, IFN-α was not induced by any of the vaccine component formulations.

The injection of the MPL-containing formulation (but not Ags and aluminum hydroxide, aluminum hydroxide alone, or Ags alone) resulted in >10 fold increases in the levels of IL-6, TNF-α, CCL2, and CCL3 compared with the PBS injection (Fig. 5A). These results indicated that MPL, and not the Ags or aluminum hydroxide, was the principal component in the vaccine that mediated the early cytokine response.

FIGURE 4. AS04 i.m. injection stimulated NF-κB activity only at the injection site. NF-κB activity was revealed by measuring luminescence using the NF-κB-luciferase transgenic mouse model. A, NF-κB activity (n = 6; measured by live imaging) at 0, 5, and 22 h after injections of AS04 in the right gastrocnemius and PBS in the left gastrocnemius. The NF-κB activity in the right hind limb is normalized to the NF-κB activity of the respective left limb. Geometric mean (●) and data points from each of the mice tested (○) are shown. Significant difference is indicated (∗) by Dunnett’s test relative to the 0 h time point.

B, NF-κB activity was measured in surgically exposed iliac, right and left inguinal lymph nodes. C, Activity measured in spleen homogenates (n = 5 donors). The measurements were taken 5 h after the injection of PBS (control), MPL or AS04 into the right gastrocnemius and PBS into the left gastrocnemius, or after the i.v. injection of MPL. All values were normalized to the respective measurement with PBS injection. Significant difference is indicated (∗) by Dunnett’s test with respect to the PBS injections. Data represent geometric mean and symbols represent the data points from each of the mice tested.
The production of cytokines was transient as the maximal levels were detected between 3 and 24 h following injection. Moreover, for a given cytokine, the peak of the MPL response was generally preceding those of the vaccine or MPL and aluminum hydroxide. Also at 24 h and 7 days, the responses induced by AS04 were typically higher than the ones induced by MPL. These results showed that aluminum hydroxide in AS04 prolonged the MPL-mediated cytokine response, especially between 6 h and 24 h.

Maximal levels CCL2, CCL3, and TNF-α were detected at 6 or 24 h after vaccine and after MPL and aluminum hydroxide injections, later than the maximal levels of IL-6, which was detected at 3 or 6 h. This suggested that the delayed dynamic of the CCL2, CCL3, and TNF-α responses reflect different regulatory pathways being activated or the additional contribution of newly recruited cells to their expression levels mainly at 24 h. The injection of aluminum hydroxide resulted in >5-fold increase in the levels of CCL2, detectable only 24 h after injection (Fig. 5A). The levels of the other cytokines were not increased to a great extent at any of the time points tested. So although aluminum hydroxide had a minor impact on some cytokine production, MPL was the main driver of the cytokine response observed in AS04.

In the serum, the i.m. injection of the vaccine resulted in a >5-fold increase in the levels of IL-6, but not of CCL2 compared with PBS (and measured between 1 h to 3 days after injection) (Fig. 5B). However, these cytokine levels were typically <10-fold lower than those found in the muscle homogenates, and were substantially much lower than those found with a systemic injection of 1 μg of MPL. The irrelevance of serum cytokines for the induction of an adaptive response was further demonstrated by evaluating the i.m. injection of one-fifth the dose of the vaccine (10 μl). The injection of 10 μl of the vaccine resulted in negligible or no changes to serum cytokine levels compared with the PBS injection (Fig. 5B). Yet injections with 10-μl doses of the vaccine gave a similar Ab response compared with injections with the full dose (50 μl) of the vaccine (Fig. 5C).

The substantially lower cytokine response detected in serum, as compared with the muscle, following i.m. injection of MPL and aluminum hydroxide or vaccine further indicated that the innate response remained local to the injection site. Furthermore, the absence of NF-κB activity in spleen with AS04 injections, also support the assumption that cytokines in the serum most likely disseminated from the local injection site rather than from de novo production in distant organs. The cytokine responses in both muscle homogenates and in serum samples were similar in BALB/c mice compared with C57BL/6 mice, suggesting that these responses are not strain-dependent (see supplemental Fig. 1). The results therefore indicated that the temporal and spatial constraints on AS04 superior adjuvant activity coincided with the transient induction of an innate immune response by AS04 acting locally at the injection site.

MPL and AS04 stimulate the infiltration of DCs and monocytes into the draining lymph node

DCs and monocytes, once activated at the site of injection by the local innate response, can migrate to the draining lymph node to provide essential information, including the antigenic determinants, to T and B cells for the generation of an adaptive response (26, 27). DC numbers and activation status were first assessed by flow cytometry in pooled draining iliac lymph...
nodes taken from mice at 6, 24, or 72 h following i.m. injection with the different components of the vaccine (Fig. 6A). At 6 h, the DC levels were similar for all of the formulations injected including PBS (data not shown). At 24 and 72 h, the injection of the MPL-containing formulation induced 3.4- to 13.5-fold higher numbers of DCs compared with the aluminum hydroxide formulations or PBS (p < 0.001) (Fig. 6A). A higher number of DCs expressing high levels of MHC class II were also observed both at 24 and 72 h with these injections (p < 0.001) (see supplemental Fig. 2). Such MHC class II<sup>high</sup> cells are indicative of recently activated DCs that have migrated from the site of injection into the draining lymph node (28, 29).

The increased expression of costimulatory molecules CD40 and CD86 provides essential signals to naive T cells to initiate an optimal adaptive response. The levels of CD40 and CD86 were quantified by flow cytometry on DCs (Fig. 6A). At 24 and 72 h, only the injection of MPL-containing formulations induced higher levels of CD40 and CD86 in the DCs as compared with the injection of PBS (p < 0.0001). Monocytes have the capacity to differentiate into DCs (27, 30) and such a differentiated population can be detected by the expression of the cell surface markers Ly6C<sup>+</sup>, CD11b<sup>+</sup>, and F4/80 (31, 32). The levels of these triple positive cells were also quantified by flow cytometry (Fig. 6A). At 24 and 72 h, the injection of MPL-containing formulations induced 3.7- to 26-fold higher numbers of monocytes compared with the aluminum hydroxide-only formulations or PBS (p < 0.05); and compared with PBS, also induced higher levels of CD40 (p < 0.05), but not of CD86.

Confirming the local induction of immunity by the vaccine or its components, the levels of CD40 or CD86 in the DCs isolated from nondraining axillary lymph nodes were not significantly increased at 24 h after injection (see supplemental Fig. 2A).<sup>7</sup> Similarly, the DCs of the spleen were also unaffected at 6 h postinjection (the anticipated time point to observe a systemic response) (see supplemental Fig. 2A).<sup>7</sup> The spleen and axillary lymph node DCs were capable of responding because the i.v. injection of MPL compared with PBS resulted in a 2-fold induction of CD40 in the axillary lymph node DCs and 2-fold induction of CD86 in spleen DCs (see supplemental Fig. 2B).

Ag-loaded cells were tracked in the draining lymph nodes using fluorescent-labeled OVA in combination with various formulations. Results generated with OVA were comparable to the ones generated previously with L1 VLP Ags (Fig. 6A). A higher DC and monocyte count per lymph node was observed at 24 h postimmunization (p < 0.05) (Fig. 6B). Hence there was no evidence that the MPL-induced innate response was affected by using a different
Ag. Moreover, higher OVA-positive DCs and monocyte numbers per lymph node (p < 0.05), expressing higher levels of CD40 and CD86 (p < 0.01) were observed with the MPL-containing formulations. These results indicated that MPL in AS04 stimulated an increased migration of Ag-loaded and activated DCs and monocytes to the draining lymph nodes at 24 h.

The direct impact of the adjuvants on DC activation and Ag uptake was further analyzed using BMDMCs stimulated in vitro with the different adjuvant formulations in combination with fluorescently labeled OVA. In a dose-dependent manner, aluminum hydroxide, MPL, or the combination of aluminum hydroxide and MPL similarly enhanced Ag uptake compared with BMDCs treated without adjuvant (Fig. 7A). However, only the MPL-containing adjuvants induced a 2-fold induction of CD86<sup>+</sup> BMDCs. Hence, the effects on in vitro Ag uptake and DC activation are also consistent with what was observed in vivo with the DCs in draining lymph nodes.

To investigate whether increased Ag uptake and costimulatory molecule expression induced by AS04 would translate into the activation of Ag-specific T cells, OVA-specific CFSE-labeled DO11.10 T cells were incubated for 72 h with BMDCs pretreated with OVA alone or in combination with MPL or aluminum hydroxide. T cell proliferation was evaluated by measuring the decrease in CFSE labeling in proliferating cells. Only the BMDMCs stimulated with MPL-containing adjuvants induced the specific proliferation of DO11.10 T cells (Fig. 7B). These results show that MPL in AS04, in contrast to aluminum hydroxide, better stimulated DCs for Ag presentation to specific T cells. Similar results were observed with the examination of BDMCs from another strain of mouse, suggesting again that this observation is not strain-dependent (see supplemental Fig. 3).

The same functional capacity of DCs was identified in vivo in draining lymph nodes 24 h after immunization. CD11c<sup>+</sup> DCs from mice injected with OVA alone, or in various formulations, were purified from pooled draining lymph nodes and incubated with CFSE-labeled DO11.10 T cells. DCs purified from mice injected with OVA/AS04 or OVA/MPL stimulated more proliferation in CD4<sup>+</sup> T cells compared with the Ag only group (p < 0.0001). Conversely, with AS04 compared with aluminum hydroxide (p < 0.005). Levels of IFN-γ, a marker of TH1 polarization, were higher with AS04 compared with aluminum hydroxide (p < 0.0001). Conversely,
the levels of IL-5, a marker of Th2 bias, were higher with aluminum salt and with the Ag alone compared with AS04 (p < 0.01). These results indicated that AS04 was a better inducer of CD4$^+$ T cell amplification and differentiation compared with aluminum hydroxide alone, and promoted a Th1 bias characteristic of a TLR4 agonist (9).

Altogether, these results demonstrated that within AS04, MPL was the principal driver of APC increased numbers and activation, and that these responses were restricted to the draining lymph node. At 24 h after injection, the results also demonstrated that MPL adsorbed on aluminum hydroxide induced a substantial wave of migrating Ag-loaded APCs to enter the draining lymph node with the enhanced capacity to directly stimulate T cells.

**Discussion**

AS04 was designed to use a well-defined immunostimulant, MPL, in combination with aluminum salt (33). The benefit of this approach has been shown by better adaptive immune responses in mice, monkeys, and humans to immunization with vaccines containing AS04 compared with the same Ags formulated with aluminum hydroxide alone (22). In this study, by using multiple approaches in vivo and in vitro, we show that the addition of MPL to aluminum hydroxide led to the rapid and spatially localized activation of an innate response, which could explain how a robust adaptive immune response is achieved in AS04-adjuvanted vaccines.

**Crucial role of MPL in AS04 for the stimulation of an innate immune response**

Our results show that MPL was responsible for the transient induction of cytokines and NF-κB activity observed within the first day following the injection of AS04, consistent with MPL acting as a TLR4 agonist (25, 34–36). MPL was also responsible for the infiltration and activation of DCs and monocytes in the draining lymph nodes by 24 h. This report is the first to our knowledge describing the local effect of MPL after i.m. immunization, the most common route used for vaccination in humans. Early induction in the muscle of NF-κB 5 h after injection was indicative of MPL directly stimulating TLR4-expressing cells. Various cell types in the muscle can express TLR4, and therefore could have been responsible for the early NF-κB activity and cytokine secretion (24, 37, 38), although infiltrating cells were likely to have also contributed at later stages. NF-κB induction was also observed in the draining lymph node at 5 h. This finding could have been due to low levels of MPL draining to the local lymph node and directly activating resident cells. Alternatively, cytokines produced in the muscle could enter the draining lymph node and in turn induced NF-κB activity. Activated cells directly migrating from the blood to the draining lymph nodes may have also contributed further to cytokine-induced NF-κB activity. Importantly, the spatial confinement of NF-κB activity to the injection site and draining lymph node was demonstrative of a localized rather than a systemic immune response to AS04 injection.

Aluminum hydroxide made little contribution to the early innate response stimulated by AS04. In addition, there was no evidence that aluminum hydroxide acted synergistically with MPL to enhance the magnitude of cytokine production (in vitro or in vivo) or to enhance the infiltration of APC in the draining lymph nodes 24 h after injection. Neither did aluminum hydroxide alter substantially the type of cytokines and recruited cells induced by MPL. However, aluminum hydroxide did prolong the cytokine responses at the injection site.

Although MPL and aluminum hydroxide could potentially cooperate in increasing IL-1β secretion (19, 20), in this study MPL stimulated IL-1β secretion independently of aluminum hydroxide in vitro. This observation is in line with a recent report by Maelfait et al. (39), which showed that LPS can induce the cleavage of pro-IL-1β. However, the authors demonstrated that this occurred via the activation of caspase-8 and not by caspase-1 as reported. Hence further work is required to evaluate the contribution of each of these caspases in the control of IL-1β secretion induced by TLR4 signaling.

In contrast to the present study, Kool et al. (30) showed that aluminum salt induced a strong and rapid inflammatory response using an i.p. route of administration. Even though a higher dose of aluminum salt was used, the discrepancy highlights the potential dependence of the adjuvant response on the route of immunization. The results in this present study are more consistent with a recent microarray analysis reporting only a few genes being altered in muscle upon aluminum injection compared with other adjuvants (40). But in common with Kool et al. (30), aluminum hydroxide stimulated Ag uptake and induced CCL2 production. These effects of aluminum salt have been linked to monocyte differentiation and migration to draining lymph nodes.

Altogether, this study suggested that the presence of aluminum hydroxide, through its recently described mode of action and its impact on cytokine response, has an overall beneficial impact on MPL response. Importantly, aluminum hydroxide also plays an important role in the physical stabilization of VLP Ags and MPL in the vaccine formulation, as well as spatially confining the Ag and MPL to the injection site.

HPV-16 and HPV-18 L1 VLPs appeared not to contribute to the early vaccine innate response compared with AS04 as shown by their inability to induce cytokine production and APC activation in vivo and in vitro. HPV L1 VLPs have been reported elsewhere to induce cytokine response in DCs, monocytes, and macrophages (41–43), possibly in a TLR-dependent fashion (44, 45). Abs against HPV L1 VLPs were found to be reduced in TLR4-deficient mice by Yang et al. (44) but not by others (46). An explanation for these discrepancies could come from the differences in the HPV L1 VLP preparation or in the experimental models used. In particular, the adsorption of HPV L1 VLPs on aluminum salt may explain their inability to stimulate innate cells.

**How does AS04 enhance Ab response compared with aluminum hydroxide alone?**

The adjuvant activity of AS04 compared with aluminum salt has been shown to induce higher levels of HPV L1 VLP-specific Abs and memory B cells in humans (22), demonstrating that AS04 is able to promote a B cell response either directly or indirectly. Recent studies clearly indicate that, contrary to murine B cells (47), human purified B cells do not express TLR4 and are not able to respond to LPS stimulation, even during concomitant BCR stimulation. Therefore this evidence does not support a role for AS04 in directly activating B cells (48–50). Similarly it is unlikely that T cells are directly activated by AS04. CD4 T cell effector functions (as measured by CD40L and IFN-γ) were not significantly induced or enhanced by MPL in this present study. In some studies, T cell responses to TLR4 agonists have been observed, such as increased adherence and Ca$^{2+}$ mobilization, but these measures are not necessarily indicators of Ag-independent T cell activation (25, 51, 52). Therefore, it is likely that the superior adjuvant activity of AS04 resulted from APC-mediated activation of T cells and subsequent B cell activation. This is supported by the rapid appearance of Ag-loaded and activated APCs in the draining lymph nodes. In such conditions, efficient Ag uptake, Ag processing, and APC-mediated presentation to T cells would be occurring at a time when the local Ag concentration is at its peak.
The cytokines induced at the injection site are functionally relevant to the cellular events detected in the draining lymph nodes. Indeed, chemokines such as CCL2 and CCL3 are known to promote the recruitment of monocytes and immature DCs (53). In particular, CCR2, the receptor for CCL2 has been shown in many models of infectious diseases and vaccination to be crucial for the mobilization of monocytes and the induction of an immune response (54–56). Differentiated monocytes and DCs are key mediators for the induction of T and B cell responses, as they both can take up Ags and migrate to the draining lymph nodes (55). In this study, the combination of MPL and aluminum hydroxide led to an optimal APC recruitment and activation in the draining lymph node, which could be due in part to aluminum hydroxide prolonging the cytokine response to MPL. The direct activation of DCs by MPL may also be crucial for a sustained Ab response. Indeed, it has been argued that the direct activation of DCs by TLR agonists enhance their ability to promote Ag-specific immunity rather than if they were activated by cytokines alone (26). IL-6 has been shown to play a role in the Ab response induced by an MPL-based vaccine (57) and this cytokine has been shown to promote Ab response by stimulating Th cells specialized in providing help to B cells (58).

This study has demonstrated that MPL in the vaccine can enhance the quality of the Ag specific T cell response. This response is associated with the rapid migration of Ag loaded and activated DCs to the draining lymph nodes. The elevated expression of CD40/86 in DCs is known to be critical for the Ag-specific activation of Th cells (59) and the optimal activation of APCs favors the local induction of Th cells expressing TCRs with the highest affinity to antigenic peptides (60). An MPL-based adjuvant has been found to specifically activate local follicular Th cells that are thought to be directly associated with the generation of B cell memory (61, 62). An appealing hypothesis is, therefore, that the superior adjuvant activity of AS04 is due to the generation in the draining lymph node of follicular Th cells with a high affinity TCR repertoire, but this remains to be determined.

Localized and transient activity of AS04 supports the favorable clinical safety profile

The localized and transient nature of the innate immune response supports the favorable safety profile for the AS04 formulated vaccines observed in humans (63, 64). In particular, proinflammatory cytokines were transiently induced and limited to the injection site, and were absent or at very low levels in the serum. Moreover, factors implicated in chronic inflammatory and autoimmune disease (65, 66) such as IFN-α induction, direct and relevant T cell activation, and sustained high cytokine levels were not observed.

Conclusion

In conclusion, this study supports a model for the mechanism of action of AS04 that can explain its advantageous adjuvant and safety properties in human vaccines. In this model, MPL in AS04 plays a central role and demonstrates that modulation of innate response by well-characterized agonists such as certain TLR agonists in an appropriate formulation can have an effective and safe way to enhance vaccine responses in humans.

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Disclosures

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