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Enhancement of Adaptive Immunity by the Human Vaccine Adjuvant AS01 Depends on Activated Dendritic Cells

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Adjuvant System AS01 is a liposome-based vaccine adjuvant containing 3-O-desacyl-4′-monophosphoryl lipid A and the saponin QS-21. AS01 has been selected for the clinical development of several candidate vaccines including the RTS,S malaria vaccine and the subunit glycoprotein E varicella zoster vaccine (both currently in phase III). Given the known immunostimulatory properties of MPL and QS-21, the objective of this study was to describe the early immune response parameters after immunization with an AS01-adjuvanted vaccine and to identify relationships with the vaccine-specific adaptive immune response. Cytokine production and innate immune cell recruitment occurred rapidly and transiently at the muscle injection site and draining lymph node postinjection, consistent with the rapid drainage of the vaccine components to the draining lymph node. The induction of Ag-specific Ab and T cell responses was dependent on the Ag being injected at the same time or within 24 h after AS01, suggesting that the early events occurring postinjection were required for these elevated adaptive responses. In the draining lymph node, after 24 h, the numbers of activated and Ag-loaded monocytes and MHCIIhigh dendritic cells were higher after the injection of the AS01-adjuvanted vaccine than after Ag alone. However, only MHCIIhigh dendritic cells appeared efficient at and necessary for direct Ag presentation to T cells. These data suggest that the ability of AS01 to improve adaptive immune responses, as has been demonstrated in clinical trials, is linked to a transient stimulation of the innate immune system leading to the generation of high number of efficient Ag-presenting dendritic cells. The Journal of Immunology, 2014, 193: 1920–1930.

Many Ags contained in subunit vaccines are inherently inert proteins or glycoproteins. A common strategy to boost Ag-specific B and T cell adaptive responses through immunization is to include an immunostimulating adjuvant in the vaccine (1–3). The ability of adjuvants to trigger early inflammatory signals, to increase Ag uptake, and to activate APCs is thought to be crucial for the efficient induction of adaptive memory immune response to vaccines (4, 5).

The Adjuvant System AS01 is a liposome-based adjuvant that contains two immunostimulants MPL and QS-21 (2). MPL is a nontoxic derivative of the LPS from Salmonella minnesota and is a TLR4 agonist (6). It can stimulate NF-κB transcriptional activity and subsequent cytokine production (6). MPL directly activates APCs such as dendritic cells (DCs) to produce cytokines and elevated levels of costimulatory molecules (7–9). QS-21 is a natural saponin molecule extracted from the bark of the South American tree Quillaja saponaria Molina (10) (reviewed in Refs. 2 and 11). The early evaluations of QS-21 as an adjuvant demonstrated that it could promote high Ag-specific Ab responses and CD8+ T cell responses in mice (12, 13) and high Ag-specific Ab responses in humans (14). The CD8+ T cell responses induced by saponin-based vaccines presumably occurred through the promotion of Ag cross-presentation in DCs (15, 16). However, specific receptors and signaling pathways induced by saponin-based adjuvants have yet to be clearly defined.

AS01 is included in a number of candidate vaccines against infections such as those caused by Plasmodium falciparum (malaria) (17, 18), varicella zoster virus reactivation (shingles) (19), HIV (20, 21), and Mycobacterium tuberculosis (22) for which both Abs and T cell immunity are thought to be involved in protection. During the preclinical and clinical evaluations of these candidate vaccines, both Ag-specific Ab and CD4+ T cell responses have been consistently observed, suggesting that AS01 promotes these adaptive responses irrespective of the type of Ag used (2). In the case of the malaria vaccine candidate, these adaptive responses have been associated with protection against malaria in various clinical trials (23–26) and have supported the selection of AS01 over other adjuvants (2, 23, 27).

Given the known immunostimulatory properties of the AS01 constituents liposome-based MPL and QS-21, the objective of this study was to describe, in mouse models, the early innate immune parameters induced by AS01 after immunization and to identify...
relationships between these parameters and the Ag-specific Ab and T cell responses raised against two model Ags, glycoprotein E [gE from varicella zoster virus (19)] and OVA.

Materials and Methods

Vaccine formulations

The Ags (clinical-grade) gE (19), Alexa Fluor 647–labeled gE, and the Adjuvant System AS01 were produced at GlaxoSmithKline. One human dose of AS01 contains 50 µg MPL (GlaxoSmithKline), 50 µg QS-21 (Antigenics, a wholly owned subsidiary of Agenus, Lexington, MA), and liposomes. OVA was obtained from Calbiochem and confirmed to be endotoxin free. Alexa Fluor 647–labeled gE and OVA were produced using the Alexa Fluor 647 Protein Labeling Kit from Invitrogen, according to the manufacturer’s instructions.

Mouse immunizations

Animal husbandry and experiments were ethnically reviewed and carried out in accordance with European Directive 2010/63/EU and the GlaxoSmithKline Biologicals Policy on the Care, Welfare and Treatment of Animals. C57BL/6 mice were purchased from Harlan Horst, and OTI and OTII mice were purchased from Charles River Laboratories. The i.m. injections were performed in 6- to 8- wk-old female mice, in both hind limbs, in either the gastrocnemius or tibialis (for histochecmistry) muscles and in a volume of 10 µl/mouse (unless stated otherwise).

Spatiotemporal relationship between AS01 and Ags on adaptive responses

Mice (n = 26/group or n = 16/group) were immunized at days 0 and 28 with different injection regimens where AS01 (1 µg QS-21 and 1 µg MPL or 5 µg QS-21 and 5 µg MPL in 25 µl), which represents 1/50th and 1/10th of the human doses of AS01, respectively, and gE (1 or 5 µg in 25 µl) were either mixed together (50 µl) or injected separately (25 µl each). Ag alone (1 or 5 µg) was injected in 50 µl. The i.m. injections were given at the same time or at separate times (with AS01 being injected from 1 h to 7 d before the Ag) at the same site or at contralateral sites into the gastrocnemius muscle.

Ag-specific Ab and T cell response

Anti-gE and anti-OVA Ab concentrations (in ELISA units per milliliter and defined by internal standards) were measured by ELISA, following a protocol described previously (19), with a minor addition: for the measurement of anti-OVA Ab concentrations, 96-well ELISA plates were coated with an overnight incubation at 4˚C of 10 µg/ml OVA in PBS. To assess Ag-specific T cell response, splenocytes from immunized mice were stimulated in vitro using 10^7 cells/ml (96-well microplate) with gE or OVA peptide pools of 15-mer with 11-aa overlap at 1.25 µg/ml (from Neosystem), respectively. Anti-CD49d and anti–CD45R/B220. Tissue sections were examined with a confocal laser scanning microscope (Zeiss LSM510/780 meta). Minor lightness and contrast adjustments were made using Zeiss and/or other routine image-manipulation software and were applied uniformly to the whole image.

Innate cell phenotyping in muscle and lymph node

Pooled tissues (gastrocnemius muscle or iliac lymph node [ILN]) from three immunized mice were first treated by mechanical dissociation in 3 ml DMEM containing Dnase 1 (100 µg/ml; Roche), 1% FCS and Liberase (Roche) at 0.1 U/ml (muscle), or 0.26 U/ml (ILN) for 30 min under agitation at 37˚C (muscle) or at room temperature (ILN). Liberase digestion was stopped by adding 10 mM EDTA and incubating on ice. Larger clumps of material were removed by passing the preparation through a 100 µm nylon cell strainer. Tissues were then homogenized and cytokines were measured in supernatants as specified (8). Briefly, the homogenates were cleared by centrifugation and stored at −80˚C until further analysis. Organs were pooled from three sets of three mice and four sets of four mice immunized with gE+AS01 or gE alone, respectively. Organs were then homogenized and cytokines were measured in supernatants as described previously (8). Brieﬂy, the homogenates were cleared by centrifugation and stored at −70˚C until analysis. Protein levels were measured by cytokine bead array (for TNF-α and IL-1β) or by cytokine-specific beads for all other cytokines using the Lumexin platform (Millipore). The levels detected for each cytokine are reported in nanograms or picograms per organ within the homogeneous supernatant of the different tissues.

Evaluation of CD11c-diphtheria toxin receptor transgenic bone marrow mouse chimeras

For the assessment of proinflammatory cytokine production, the right gastrocnemius muscle and right iliac lymph node were collected after immunization at the indicated time points and stored at −80˚C until further analysis. Organs were pooled from three sets of three mice and four sets of four mice immunized with gE+AS01 or gE alone, respectively. Organs were then homogenized and cytokines were measured in supernatants as described previously (8). Briefly, the homogenates were cleared by centrifugation and stored at −70˚C until analysis. Protein levels were measured by cytokine bead array (for TNF-α and IL-1β) or by cytokine-specific beads for all other cytokines using the Lumexin platform (Millipore). The levels detected for each cytokine are reported in nanograms or picograms per organ within the homogeneous supernatant of the different tissues.

Cytokine detection in muscle and dLN

Fluorescence-activated cell sorting (FACS) analysis

To assess Ag-specific T cell response, splenocytes from immunized mice were stimulated in vitro using 10^7 cells/ml (96-well microplate) with gE or OVA peptide pools of 15-mer with 11-aa overlap at 1.25 µg/ml (from Neosystem) and incubated for 72 h. Large cell populations were isolated from OTI and OTII transgenic mice, respectively, and stained with the following anti-mouse Abs: anti-Ly-6G-FITC, the PE-conjugated lineage identifiers (anti-SiglecF, anti-CD16b, anti-CD4, anti-CD11c, and anti–NK1.1), anti-CD49d-PE, anti–Ly-6G-PerCP, anti–Ly-6C-PerCP, anti–CD3-APC-Cy7, anti–CD64-APC-Cy7, and anti–CD40-PE. All Abs were obtained from BD Biosciences. In the experiments described previously (13), the QS-21-sulfo-N-hydroxy succinimide derivative was purified by precipitation with ethyl acetate, and dicyclohexylcarbodiimide, as described previously (13). The QS-21-sulfo-NH2-ester derivative was purified by precipitation with ethyl acetate, then dried and suspended in 0.1 M NaHPO4 buffer (pH 7), and reacted with a volume of 10 µg/ml OVA in PBS. To label QS-21, an Alexa Fluor 647–labeled gE and OVA were produced using the Alexa Fluor 647 Protein Labeling Kit from Invitrogen, according to the manufacturer’s instructions.

Imaging

Fluorescent AS01 was prepared by labeling QS-21 with the fluorophore BODIPY (4,4-difluoro-4-bora-3a,4a diaza-s-indacene). To label QS-21, an Alexa Fluor 647–labeled gE and OVA were produced using the Alexa Fluor 647 Protein Labeling Kit from Invitrogen, according to the manufacturer’s instructions.
using a FACSria (>95% purity) from ILNs of mice previously immunized (24 h before) with OVA (5 μg)/AS01 in each hind leg. OT-I and OT-II proliferation was determined by the loss of CFSE fluorescence by flow cytometry. As controls, APCs were incubated with MHCI- or MHCII-restricted OVA peptides for 45 min at 37˚C, washed, and incubated with $25 \times 10^3$ OT-I and OT-II, respectively, at various APC:T ratios as indicated, starting with 1:2 ratio.

Statistical analyses

All statistical analyses on cytokine concentrations, Ab concentrations, and T cell frequencies were performed on logarithmic transformed data. The Shapiro–Wilk test was used to evaluate normality. An ANOVA followed by the Tukey’s or Dunnett’s test was applied to identify differences between treatment groups (see figure legends). Statistical significance was assigned when $p \leq 0.05$, and in addition for the analysis of Ab concentrations, a condition for which the difference between the groups was also $>2$-fold was applied. Principal component analysis was used to identify correlations between cytokine and immune cell responses to immunization. Absolute quantities and fold differences of repeated measures described in Results are derived from geometric means. Principal component analysis was performed with SPAD software (version 7; Coheris, Suresnes, France) All other analyses were performed using SAS software (version 9.2; SAS Institute).

Results

Localization of the vaccine after i.m. injection

Fluorescently labeled QS-21 and Ag were incorporated into the gE/AS01 vaccine to monitor the vaccine’s distribution after i.m. injection in situ (Fig. 1). In the muscle, 30 min after injection, QS-21 and the Ag were located in interstitial regions between muscle fibers, mainly in the perimysium (Fig. 1A). At 3 and 24 h, QS-21 and Ag were detected at much lower levels than at 30 min suggesting a rapid clearance from the muscle. Both QS-21 and Ag were also detected in the dLN as early as 30 min, suggesting a rapid drainage from the injection site to the dLN (Fig. 1B). At

![Figure 1](http://www.jimmunol.org/DownloadedFrom)
30 min, QS-21 was mainly detected in the subcapsular region of the dLN, whereas at 24 h, it was also detected in the medulla. In contrast to QS-21, the Ag was detected deep within the cortical and medullary sinuses at 30 min but was barely detectable at 24 h at the resolution used in this study. Although these data should be interpreted with caution because QS-21-BODIPY may not fully reflect the behavior of unlabeled QS-21 and the whole AS01 formulation, the differences in the localization and dynamics of QS-21 and Ag suggested that the two components were not physically associated and had different pharmacokinetic properties. This concurred with biochemical analyses, which indicated that AS01 was indeed not physically associated with the Ag in the vaccine formulation (data not shown). Because MPL and QS-21 are included in the same liposome, it is possible that MPL has a similar distribution as QS-21, but this was not assessed in this study.

**Rapid and transient induction of cytokines by AS01 at the injection site and dLN**

The kinetics of the innate immune response was examined by measuring a panel of representative proinflammatory cytokines and chemokines, as well as the main innate immune cell subset in the muscle and dLN, over a period of 7 d after the injection of gE/AS01 or gE.

The peak induction of all the cytokines considered together typically occurred within 24 h after gE/AS01 injection and was substantially greater (≥8.7-fold) than after Ag injection alone (Fig. 2A). The overall cytokine concentration in the muscle was higher (up to 16-fold at 16 h) than in the dLN. The rapid induction of cytokines by 3 h at the muscle injection site and dLN suggested that cytokines in both locations were induced by resident cells directly stimulated by AS01. This is consistent with a rapid drainage of AS01 from the injection site to the dLN as suggested by the confocal imaging (see Fig. 1). The cytokine levels declined by 48 h and returned to baseline at day 7 (= 168 h), demonstrating the transient nature of the response (Fig. 2A, 2B). More specifically, the pattern of cytokines production was dominated by chemokines rather than proinflammatory cytokines, with monocyte and granulocyte-attracting chemokines (e.g., CCL2 and CXCL1, respectively) and T cell–attracting IFN-dependent CXCL9 and CXCL10. Except for IL-6, the other proinflammatory cytokines (e.g., IL-12p70 and IL-1β) were only induced at low levels (<80 pg/organ). Although most cytokines peaked at early time points (either 6 or 24 h) and subsided rapidly (e.g., CXCL1 and IL-6), others subsided only between days 2 and 7 (e.g., CXCL9 and CCL2) (Fig. 2A, 2B). Furthermore, neither IL-12p70 nor IFN-α was detected above baseline levels (data not shown). The pattern of the inflammatory response was similar overall between muscle and dLN, except for CXCL9 and CXCL10, which were more rapidly increased at 3 h in the dLN than in the muscle.

These data showed that AS01 induced an inflammatory response at the injection site and dLN, characterized by the production of chemokines and IFN-pathway–related cytokines. This inflammatory response was transient and subsided by 7 d.

**AS01 promotes the transient recruitment of innate cells in the muscle and dLN**

Immune cell numbers were evaluated by enzymatic digestion of muscle injection sites and dLNs, followed by cell phenotyping using flow cytometry (Fig. 3).

In the muscle and consistent with the chemokine profile (see Fig. 2), monocyte (Lin− CD11b+Ly6C<sub>high</sub>) and neutrophils (SSC<sub>high</sub> Ly6G<sup>+</sup>) were the most prominent immune cell types whose numbers were transiently elevated after the injection of gE/AS01 compared with Ag alone (Fig. 3A, 3B). Lymphocytes numbers were relatively low during the period investigated. At 3 h, the number of neutrophils was 9.5-fold higher with gE/AS01 than with Ag alone, and at 6 h, the numbers of monocytes and neutrophils peaked and were 4.9- and 13-fold higher, respectively, with gE/AS01 than with Ag alone. In comparison, the number of DCs (Lin+ CD11c+MHCII<sup>+</sup>) was low with no significant changes during the period investigated. At day 7, the respective numbers of immune cells were not different after the injection of gE/AS01 or Ag alone, suggesting that the local inflammatory reaction at the injection site fully resolved by 7 d.

In the dLN and at 6–48 h, the total number of immune cells was greater after the injection of gE/AS01 than after Ag alone.
FIGURE 3. AS01 induced a transitory increase in innate immune cell numbers in the muscle (A and B) and dLN (C and D), 3–168 h (= day 7) postimmunization of AS01 (1 µg MPL and 1 µg QS-21)-adjuvanted or unadjuvanted (No adj.) gE-Alexa Fluor 647 (2 µg) Ag. (A–C) Geometric mean cell number/tissue (n = 3) of (CD45+) immune cells including monocytes (Lin–CD11b+Ly6Chigh), neutrophils (SSChighLy6G+), and DCs (CD11c+MHCIIhigh in muscle and CD11c+MHCIIhigh in the dLN). (E) The immune cell and cytokine kinetics (shown in Fig. 3) were examined using principal component analysis (PCA). The principal components 1, 2, and 3 (represented by the x, y, and z axes, respectively, in the three-dimensional scatter plot) accounted for 73 and 88% of the variation in the injection site (muscle; upper scatter plot) and dLN (lower scatter plot) data sets, respectively. Neutrophils (N) are depicted by cyan triangles. Other cell types are depicted by gray triangles (B, B cells; mDC, MHCIIhigh DCs; rDC, MHCIImed DCs; M, monocytes; and T&NK, T and NK cells). Cytokines clusters with potential associations with neutrophils are depicted by purple circles (see also Supplemental Table I) and include (IL-6, IL-1β, TNF-α, (Fig. 3C, 3D). A 3.4- to 4-fold increase in lymphocytes was observed as early as 3 h after injection. In contrast to the muscle, the number of myeloid cells only represented a minority of the total population (1–7%) in the dLN. However, the numbers of neutrophils, Ly6C<sup>high</sup> monocyte, and DCs (defined as CD11<sup>c+</sup> and MHCII<sup>high</sup> cells, furthermore defined as MHCII<sup>high</sup> DCs) were higher by up to 53-, 230-, and 8.6-fold, respectively, after the injection of gE/AS01 compared with Ag alone (Fig. 3D). The early increase in the number of neutrophils at 3 h was consistent with the rapid appearance of neutrophil chemoattractants (CXCL1; see Fig. 2). At day 7, lymphocytes accounted for the remaining increase in cell numbers in the dLN, whereas myeloid cell numbers returned to baseline levels. The increase in the number of DCs was apparent from 16 h and consistent with the migration of muscle resident DCs into the dLN observed by Langlet et al. (29) after the i.m injection of alum.

Multicomponent analysis was further performed to correlate the kinetics of the various cytokines with cell numbers (Fig. 3E, Supplemental Table I). Neutrophil kinetics in the muscle was associated (correlation coefficient r > 0.5) with a number of cytokines, including IL-6, TNF-α, and IL-1β, as well as two known neutrophil-attracting cytokines (CXCL2 and CXCL11), suggesting that early proinflammatory signals were associated with rapid neutrophil migration to the injection site. However, in the dLN, neutrophil kinetics was associated with a different but overlapping set of cytokines than in the muscle, suggesting a different mechanism regulating the increase in neutrophil number in the dLN. In contrast to neutrophil kinetics, the kinetics of the other immune cell types in the muscle and dLN were not so clearly associated with any of the cytokines examined, suggesting that the behavior of these cells was governed by a more complex pattern of signals. In particular, monocyte recruitment did not seem to be associated with CCL2 in this model.

**Spatiotemporal colocalization of AS01 and the Ag is necessary for adjuvant effect**

To investigate the time window in which the kinetics of the innate immune responses relate to the adaptive immune responses, AS01 was first injected followed by gE at different intervals [from 0 to 72 h at the same location in the muscle (Fig. 4, left graphs)]. Two weeks after immunization, Ag-specific Ab and T cell responses were measured. When the Ag and AS01 were injected at the same time in the same site, either separately or premixed, the respective Ab and T cell responses were not different. However, these Ab and T cell responses were >13,000- and >5.5-fold higher, respectively, than after the injection of Ag alone. The adaptive immune responses were also high when the Ag was injected 1 h after AS01 instead of at the same time. However, the adaptive responses were substantially lower when the Ag was injected 24 or 72 h after AS01 (Ab responses, 6- and 540-fold lower, respectively, and T cell responses, 5.3- and 6-fold lower, respectively). With the 72-h separation between injections, the T cell response was not different from that induced by Ag alone, although the Ab response was 24-fold higher than that induced by Ag alone. However, this difference in Ab responses was not observed when 5-fold lower amounts of AS01 and Ag were used (Supplemental Fig. 1).

To investigate the spatial relationship between the innate immune responses and the adaptive responses, the Ag was injected in the limb contralateral to that in which AS01 was injected (Fig. 4, right graphs). Ag-specific Ab and T cell responses were
FIGURE 4. High Ab and T cell responses were dependent on the Ag being injected at the same site and within 24 h after AS01. Ag-specific Ab concentrations (upper graphs; \( n = 26 \), left; \( n = 16 \), right) and CD4\(^+\) T cell responses (percentage of gE-specific CD4\(^+\) T cells with respect to all CD4\(^+\) T cells; lower graphs; \( n = 13 \), left; \( n = 8 \), right) in splenocyte cultures derived from mice subject to different injection regimes. The cytokine expression profile (IL-2, IFN-\(\gamma\), or both) of the gE-specific CD4\(^+\) T cells are indicated by differences in shading. The values from mice injected with AS01 and Ag together in the same syringe are indicated by hatched bars. The adaptive responses were measured 30 d after the second immunization. The Ag was 5 \( \mu \)g E and AS01 included 5 \( \mu \)g MPL and 5 \( \mu \)g QS-21. Histograms represent geometric means, and error bars represent 95% confidence intervals. Highlighted statistical relationships are indicated by horizontal lines, either between the coinjection of AS01 and Ag and other regimes (black lines) or between the injection of Ag alone and other regimes (gray lines). ***\( p < 0.001\).

*substantially lower (110- and 4.4-fold, respectively) when gE was injected in the contralateral site rather than the same site as AS01 (Fig. 4, *right graphs*). Although the T cell response was not different from that when Ag was injected alone, the Ab response was slightly higher. However, this difference in Ab responses was not observed when 5-fold lower amounts of AS01 and Ag were used (Supplemental Fig. 1).

These results indicated that maximal Ag-specific T cell and Ab responses required the Ag to be injected at the same site together with AS01 and within 24 h. This is consistent with the rapid clearance of Ag and adjuvant at the injection site, as shown by confocal imaging (Fig. 1). This also suggested that elements of the innate immune response that were triggered by AS01 within 24 h and local to the injection site potentially contributed to the increased magnitude and quality of the adaptive responses. This limited time window seemed to be more critical for the induction of T cell response than for Ab response.

**AS01 enhances the recruitment and activation of APCs in the dLN**

The increased activity of APCs in the dLN could explain how the activation of innate immunity by AS01 translates into enhanced Ag-specific adaptive immune responses. Ag-carrying innate immune cells were identified using fluorescently labeled gE Ag, even though the method could have underestimated their number because of the anticipated loss of fluorescence with Ag degradation after uptake (Fig. 5A, 5B). Higher numbers of Ag\(^+\) monocyte, MHCII\(^{\text{high}}\) DCs, and neutrophils were detected up to 48 h after immunization with gE/AS01 than with Ag alone (Fig. 5A). At early time points, neutrophils were the main Ag-loaded cells, but their numbers rapidly subsided. After 24 h, Ag\(^+\) monocytes were 10 times more frequent than Ag\(^+\)MHCII\(^{\text{high}}\) DCs. Also Ag\(^+\) monocytes were 22% of all monocytes, whereas Ag\(^+\)MHCII\(^{\text{high}}\) DCs were 4% of all MHCII\(^{\text{high}}\) DCs. In contrast to other adjuvants such as alum or emulsion in the same model (8, 30), AS01 did not increase the amount of Ag detected per cell as measured by mean fluorescence intensity of Ag\(^+\) cells (Fig. 5B). However, monocyte and MHCII\(^{\text{high}}\) DCs expressed higher levels of the costimulatory molecule CD86 and CD40 at 24 h after the injection of gE/AS01 than after Ag alone (Fig. 5C). The expression of CD40 and CD86 returned to baseline levels at day 7, consistent with the transient nature of the AS01-dependent innate immune response. Therefore, coadministration of AS01 with the Ag increased the number of Ag-carrying and activated APCs without a clear increase in their capacity to take up Ag.

Both DCs and monocyte populations are heterogeneous (Fig. 5D) (31, 32). At 24 h after immunization with gE/AS01, a substantial proportion of Ly6C\(^{\text{high}}\) monocytes expressed CD11c\(^+\) and MHCII, showing that AS01 affected the differentiation of monocytes in situ. The MHCII\(^{\text{high}}\) DCs population contained cells that expressed CD11b and CD64 that are both markers of monocyte-derived DCs (33), suggesting that, in addition to the classical CD24\(^+\) DCs (also known as CD8a\(^{+}\) DCs) and CD11b\(^+\) CD64\(^{\text{high}}\) DCs, a proportion of DCs were derived from monocytes (29, 34). Given that those Ly6C\(^{\text{high}}\) CD64\(^{\text{low}}\) monocyte-derived DCs were part of the MHCII\(^{\text{high}}\) population (see Fig. 5D), they expressed higher levels of MHCII than Ly6C\(^{\text{high}}\) monocytes. This transition of monocytes into DCs was more apparent in the dLNs from mice injected with gE/AS01 than with gE alone (i.e., 13% compared with 4% of CD11b\(^+\)CD64\(^{\text{low}}\) cells in the Ly6C\(^{\text{high}}\) CD11c\(^{+}\) MHCII\(^{\text{high}}\) population). Whether these differentiated monocytes originated from the muscle after extravasation or directly from blood was not assessed.

In the dLN after the injection of gE alone, the CD11c\(^+\) DCs could also be resolved into two distinct populations by the relative expression level (medium or high) of MHCII. The MHCII\(^{\text{medium}}\) population (Fig. 5D, asterisk in nonadjuvant group) reflected a LN-resident DC population that was not apparent 24 h after the injection of gE/AS01, suggesting that AS01 may have directly induced higher levels of MHCII in this population. Therefore, AS01 broadened the heterogeneity of MHCII\(^{\text{high}}\) DCs population in the dLNs to contain bona fide DCs (either resident or migrating from the muscle injection site) and monocyte-derived DCs.

**Improved T cell response by AS01 is mediated by MHCII\(^{\text{high}}\) DCs**

To examine the ability of the different APC subsets to present Ag to cognate T cells, two murine models were used (5): one that measured responses after DT-mediated CD11c\(^+\) cell depletion and a second that measured the ability of purified APC population to present OVA to cognate–T cell ex vivo. OVA was confirmed as a suitable model Ag because i.m. immunization with OVA/AS01 compared with OVA alone increased OVA-specific CD4\(^+\) and CD8\(^+\) T cell frequencies (Supplemental Fig. 2A). Restimulation with OVA peptide pools of splenocytes from OVA/AS01-immunized mice also indicated that both Th1 (IFN-\(\gamma\)) and Th2 cytokines (IL-5 and IL-13) were induced, although the cytokine response was largely shifted toward Th1 compared with OVA/Alum-immunized mice (Supplemental Fig. 2B).

CD11c\(^+\) cell depletion was achieved by injecting DT into animals that had bone marrow transplants from mice transgenic for...
DTR driven by the CD11c promoter (35). Transgene expression rendered the cells sensitive to the lethal effect of DT.

Using an immunization protocol combining both Ags, gE and OVA, gE-specific and OVA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were higher with gE-OVA/AS01 than with gE-OVA alone (Fig. 6A), in line with the other immunizations in wild type (WT) mice (see Fig. 4, Supplemental Fig. 2A, 2B). The injection of DT 12 h before gE-OVA/AS01 immunization abrogated both CD4<sup>+</sup> and CD8<sup>+</sup> OVA and gE-specific responses (Fig. 6A), indicating that CD11c<sup>+</sup> cells were necessary for the induction of the Ag-specific T cell responses to vaccines adjuvanted with AS01.

Because both MHCII<sup>high</sup> DCs and a subset of Ly6C<sup>+</sup> monocytes express CD11c<sup>+</sup> (see Fig. 5D), the ability of those two APC subsets to directly present Ag (OVA) to T cells was assessed ex vivo using OTII and OTI T cells. Cells were isolated by cell sorting from dLNs 24 h after immunization with gE/AS01 (Supplemental Fig. 2C, 2D). As shown for the gE Ag in Fig. 5A, the proportion of OVA<sup>+</sup> cells after injection of fluorescent OVA/AS01 had
been found to be higher in the monocyte population than in the DC population (25–34 versus 4–9%). OTII- or OTI-proliferation after 3 d of coculture (measured by the decrease of the CFSE fluorescent cytosolic dye) was used as a readout of Ag presentation. MHCII\textsuperscript{high} DCs from mice immunized with OVA/AS01 substantially induced both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell proliferation in a T cell:DC ratio dependent manner (Fig. 6B, 6C). In contrast, Ly6C\textsuperscript{+} monocytes (which excludes monocyte-derived DCs) from mice immunized with OVA/AS01 did not induce significant CD4\textsuperscript{+} or CD8\textsuperscript{+} T cell proliferation at any ratio. Pulsing these monocytes with OVA peptides restored CD8\textsuperscript{+} but not CD4\textsuperscript{+} T cell proliferation (data not shown), demonstrating that these cells were not able to present Ag to CD4\textsuperscript{+} T cells, even in the presence of excess peptides loaded onto MHCII molecules.

This suggests that AS01 enhanced the ability of MHCII\textsuperscript{high} DCs to present Ag to T cells but not monocytes, even though monocytes were the main cell type carrying Ag in the dLN (see Fig. 5A), and expressed MHCII and costimulatory molecules (see Fig. 5C, 5D, Supplemental Fig. 2C).

Therefore, because AS01 increased the number of MHCII\textsuperscript{high} DCs in the dLN, as well as their ability to efficiently prime T cells, these DCs were likely to be responsible for most of the T cell response after immunization with an AS01-adjuvanted vaccine. T cell activation did not appear to require an increase in Ag loading per se but was associated with the increased activation of DCs, as suggested by the elevated expression of CD40 and CD86. Although Ly6C\textsuperscript{+} monocytes were unable to efficiently activate T cells, DCs derived from monocytes (Ly6C\textsuperscript{+}CD11c\textsuperscript{+}MHCII\textsuperscript{high}CD11b\textsuperscript{+}CD64\textsuperscript{+}) that are included in the MHCII\textsuperscript{high} DC population may have contributed to T cell priming.

Discussion

Adjuvant System AS01 contains two immunostimulants, MPL and QS-21, in a liposome formulation. After its injection, the expected impact on the innate immune response was clearly demonstrated in the mouse models used in this study. This innate immune response was rapid and transient in both the injected muscle and dLN, and reflected to a large degree, the rapid drainage of the Ag and the adjuvant from the muscle to the dLN. The significance of this innate response was supported by the observation that the enhanced Ag-specific B and T cell responses were largely dependent on the Ag being injected at the same location as AS01 within 24 h. Several innate immune parameters induced by AS01 may explain its ability to promote adaptive immune responses.

First, AS01 increased the number of APCs that were efficient at priming T cells in the dLN. These APCs were within the MHCII\textsuperscript{high} DC population and were identified in ex vivo presentation assays and in an in vivo transgenic CD11c\textsuperscript{+} cell depletion model. Among other mechanisms, the activation of this DC population, reflected by elevated expression levels of costimulatory molecules, may have directly contributed to T cell–priming efficiency (36, 37). However, unlike other adjuvants such as oil-in-water emulsions, MPL, or alum-containing adjuvant AS04 (8, 30, 38), AS01’s adjuvant effect was not associated with an increased uptake of Ag by

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**FIGURE 6.** T-cell responses to immunization were dependent on CD11c\textsuperscript{+} MHCII\textsuperscript{high} DCs. (A) Geometric mean Ag-specific T cell responses (n = 6/group) 7 d after the second immunization with gE + OVA or gE + OVA/AS01 (OVA, 1 μg; gE, 5 μg; and AS01 included 5 μg MPL and 5 μg QS-21) in wild type mice containing bone marrow transplants from transgenic (CD11c-DTR) mice and treated with (+) or without (−) injection of DT 24 h before immunization. (B) Representative flow cytometry histograms of OTII CD4\textsuperscript{+} T cell and OTI CD8\textsuperscript{+} T cell activation measured in terms of loss of cell cytosolic CFSE fluorescence (because of proliferation) after ex vivo coculturing with DCs (CD11c\textsuperscript{+}MHCII\textsuperscript{high}) or monocytes (CD11b\textsuperscript{+}Ly6c\textsuperscript{+}) taken 24 h after immunization with OVA/ AS01 (5 μg OVA and AS01 included 1 μg MPL and 1 μg QS-21) in different ratios starting at two T cells to one APC. Green and purple areas under the curves denote proliferating and nonproliferating cell populations, respectively. (C) Quantification of OT-II and OT-I cell divisions with respect to different ratios of T cells to APCs.
DCs or with an Ag depot effect. The DC population included tissue-resident DCs that migrated to the dLN upon activation and monocyte-derived DCs, similar to what has been shown before with a LPS adjuvant, as well as with alum adjuvant injected via the peritoneal route (5, 29, 39). Indeed, MPL in AS01 may have directly activated muscle-resident DCs (8) and could also have enhanced the differentiation and migration of monocyte-derived DCs (29).

Second, the rapid and concomitant free-flow of AS01 and Ag to the dLN was likely to have affected the magnitude of the adaptive response. Resident DCs in the dLN may have been directly activated by AS01 (or indirectly by the inflammatory milieu) and may therefore provide an early signal to cognate T cells, before the accumulation of the migratory DCs. The rapid activation of DCs at a stage when the Ag was most readily available to be endocytosed (i.e., soon after immunization) may be an additional key factor in establishing a strong T cell response. This is suggested by Kamath et al. (40) who recently demonstrated that, in the converse situation, the early recruitment of nonactivated Ag-loaded APCs with in the dLN may be detrimental for Th1 responses. Given that high adaptive responses were achieved even when Ag was injected hours after AS01, our study shows that high adaptive responses can be associated with a rapid induction of inflammation and APC activation and may not require a physical association between adjuvant and Ag.

Third, in the dLN, the rapid recruitment of naïve T cells was likely to have further enhanced the frequency of T and APC interactions (41), thereby supporting Ag presentation. This may be explained by the early activation of resident DCs in the dLN, as shown by others (42). The CXCR3 ligands CXCL9 and CXCL10 are often associated with Th1 responses (43) and were prominent among the cytokines detected in the dLN. The expression of both chemokines by LN stromal cells and DCs may have enhanced Th1 responses by creating a favorable environment facilitating T cell priming at the periphery of the dLN (44).

Fourth, other innate immune effectors also may have modulated the adaptive response. Neutrophils were rapidly recruited at the injection site and in the dLN after immunization with AS01-adjuvanted vaccine. Although not assessed in this study, neutrophils have been reported to have either no effect or a detrimental effect on the adaptive response (45, 46). Nevertheless, neutrophils may play a role in controlling the degree of local inflammation and participate in Ag clearance (47). Monocytes were the largest population of innate immune cells recruited after immunization with gE/AS01. Monocytes may play a critical role in the resolution of inflammation at the injection site by differentiating locally into macrophages (48). This is supported by the absence of inflammatory cells and cytokines in the muscle 1 wk post-immunization with gE/AS01, suggesting a full recovery of the injected muscle. Although some of the Ly6c<sup>high</sup> monocytes expressed MHCIi and costimulatory molecules and were loaded with Ag, most of these cells were unable to prime T cells efficiently, in line with previous reports (29, 49, 50). An immunomodulatory role of those monocytes in AS01 mode of action remains to be investigated. Indeed, monocytes may have supportive roles, such as helping DCs in directing Th1 differentiation of CD4<sup>+</sup> T cells (51) or in activating CD8<sup>+</sup> memory T cells and NK cells (52), but may have had also a suppressive role, as reported recently (53).

Both MPL and QS-21 in AS01 have been associated with enhancing Ab and T cell responses after immunization (19) and may affect the innate immune responses differently. In our study, the induction of a number of cytokines (e.g., IL-6, CCL2, and CCL3) may have been dependent on MPL (8), whereas the induction of IL-1β may have been due to QS-21 because IL-1β induction is associated with the activation of the inflammasome, and the activation of the inflammasome has been observed with QuilA (54). In addition, QS-21 is likely to have played a role in CD8<sup>+</sup> T cell induction in the mouse model used in this study, perhaps mediated by a particular population of DCs, as reported recently (35). The contributory roles of MPL and QS-21 in the response are currently under investigation.

AS01 has been selected in a number of human vaccine candidate dates because of its association with enhanced immune responses, both humoral and cellular (2, 19, 55). Recently, the RTS,S vaccine was shown to confer protection of 30–47% against uncomplicated and severe malaria in young African children and infants, respectively (17, 18). Although one difference between mice and human is the lack of detectable CD8<sup>+</sup> T cell responses in humans (21, 22, 25), the results described here now provide insights into the role of AS01 in generating protective vaccine responses in humans, particularly through its ability to generate a strong cellular response. The features of AS01’s adjuvant activity that are likely to be relevant in humans are the transient activation of an innate response at the site of injection, the induction of a specific pattern of cytokines, and the generation of a heterogeneous DC population in the dLN that is efficient at priming T cells. Hence, the details on AS01’s adjuvant activity identified in this study may help in the further development of human vaccine adjuvants required for promoting Ag-specific cell-mediated responses as well as Ab responses.

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Disclosures


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